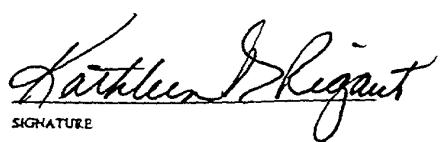


U. S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE (REV 5-93)		ATTORNEY'S DOCKET NUMBER UNMC 63117B
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (REDOCKED OR 37 CFR 1.57) 09/445653 not yet assigned
INTERNATIONAL APPLICATION NO PCT/US 98/12138	INTERNATIONAL FILING DATE 11 June 1998	PRIORITY DATE CLAIMED 13 June 1997
TITLE OF INVENTION COMPOSITIONS FOR DELIVERY OF BIOLOGICAL AGENTS AND METHODS FOR THE PREPARATION THEREOF		
APPLICANT(S) FOR DO/EO/US KABANOV, Alexander V.; EISENBERG, Adi; KABANOV, Victor A.		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information		
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</p> <p>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p>		
Items 11. to 16. below concern other document(s) or information included:		
<p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p>		
<p>13. <input type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>14. <input type="checkbox"/> A substitute specification.</p> <p>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>16. <input checked="" type="checkbox"/> Other items or information: Small entity statement Copy of form PCT/IB/308</p>		

420 Rec'd PCT/PTO 10 DEC 1999

U.S. APPLICATION NO. 09/144,965 not assigned		INTERNATIONAL APPLICATION NO. PCT/US98/12138	ATTORNEY'S DOCKET NUMBER UNMC 63117B																
17. <input checked="" type="checkbox"/> The following fees are submitted:		CALCULATIONS PTO USE ONLY																	
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO.....  International preliminary examination fee paid to USPTO (37 CFR 1.482)  No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))..  Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....  International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)..... 																			
ENTER APPROPRIATE BASIC FEE AMOUNT =		\$ 96.00																	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		\$ 130.00																	
<table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th>Claims</th> <th>Number Filed</th> <th>Number Extra</th> <th>Rate</th> </tr> </thead> <tbody> <tr> <td>Total Claims</td> <td>18 - 20 =</td> <td>0</td> <td>X 78 \$ 0</td> </tr> <tr> <td>Independent Claims</td> <td>1 - 3 =</td> <td>0</td> <td>X 18 \$ 0</td> </tr> <tr> <td colspan="2">Multiple dependent claims(s) (if applicable)</td> <td>+</td> <td>\$</td> </tr> </tbody> </table>		Claims	Number Filed	Number Extra	Rate	Total Claims	18 - 20 =	0	X 78 \$ 0	Independent Claims	1 - 3 =	0	X 18 \$ 0	Multiple dependent claims(s) (if applicable)		+	\$	TOTAL OF ABOVE CALCULATIONS = \$ 226.00	
Claims	Number Filed	Number Extra	Rate																
Total Claims	18 - 20 =	0	X 78 \$ 0																
Independent Claims	1 - 3 =	0	X 18 \$ 0																
Multiple dependent claims(s) (if applicable)		+	\$																
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).		\$ 113.00																	
SUBTOTAL =		\$ 113.00																	
Processing fee of \$130.00 for furnishing the English translation later the <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).		+ \$ 0																	
TOTAL NATIONAL FEE =		\$ 113.00																	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +		\$ 0																	
TOTAL FEES ENCLOSED =		\$ 113.00																	
		Amount to be: refunded \$ charged \$																	
a. <input checked="" type="checkbox"/> A check in the amount of \$ 113.00 to cover the above fees is enclosed.  b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.  c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 04-1406. A duplicate copy of this sheet is enclosed.																			
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.																			
SEND ALL CORRESPONDENCE TO:  Kathleen D. Rigaut, Ph.D., J.D. DANN, DORFMAN, HERRELL AND SKILLMAN 1601 Market Street, Suite 720 Philadelphia, Pennsylvania 19103																			
 SIGNATURE Kathleen D. Rigaut, Ph.D., J.D. NAME 43,047 REGISTRATION NUMBER																			

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant or Patentee: Kabanov et al.

Application or Patent No.: Not Yet Assigned

Filed or Issued: Concurrently Herewith

For: Compositions for delivery of biological agents and methods for the preparation thereof

**VERIFIED STATEMENT (DECLARATION) SUPPORTING ANOTHER'S CLAIM FOR  
SMALL ENTITY STATUS 37 CFR § 1.9(f) AND § 1.27(d)(1) - NONPROFIT ORGANIZATION**

I hereby declare that I am making this verified statement to support a claim by the above-identified applicant or patentee for small entity status for purposes of paying reduced fees with regard to the above-identified invention described in

the specification filed herewith  
 International Application, filed 11 June 1998 PCT/US98/12138  
 U.S. Patent No. \_\_\_\_\_, issued \_\_\_\_\_

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

FULL NAME OF ORGANIZATION:

BOARD OF REGENTS OF THE  
UNIVERSITY OF NEBRASKA

TYPE OF ORGANIZATION

University or other institution of Higher education  
 Tax exempt under U.S. Internal Revenue Code 26 USC § 501(a) and  
 Nonprofit scientific or educational under statute of U.S.A.

ADDRESS OF ORGANIZATION:

Regents Hall  
3835 Holdrege Street  
Lincoln, NE

Name of State:

Citation of Statute:

Would qualify as tax exempt under U.S. IRC if located in U.S.A.  
 Would qualify as nonprofit scientific or education under statute of  
state of U.S.A if located in U.S.A.

Name of State:

Citation of Statute:

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR § 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States code to the above-identified invention.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization known to have rights to the invention is listed below\* and the organization knows of no rights to the invention being held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR § 1.9(c) if that person had made the invention, or by any concern which would not qualify as a small business concern under 37 CFR § 1.9(d) or by a nonprofit organization under 37 CFR § 1.9(e).

FULL NAME:

ADDRESS:

INDIVIDUAL  SMALL BUSINESS CONCERN  NONPROFIT ORGANIZATION

FULL NAME:

ADDRESS:

INDIVIDUAL  SMALL BUSINESS CONCERN  NONPROFIT ORGANIZATION

FULL NAME:

ADDRESS:

INDIVIDUAL  SMALL BUSINESS CONCERN  NONPROFIT ORGANIZATION

\* NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention asserting to their status as small entities. (37 CFR § 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 CFR § 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: David A. Crouse, PhD

University of Nebraska Medical Center,

Title in Organization: Vice Chancellor, Academic Affairs Board of Regents  
Address: 600 South 42nd Street, Box 986810, Omaha, NE 68198-6810

Signature: *David A. Crouse* Date: *12/10/98*

COMPOSITIONS FOR DELIVERY OF  
BIOLOGICAL AGENTS  
AND METHODS FOR THE PREPARATION THEREOF

Alexander V. Kabanov  
Adi Eisenberg  
Victor A. Kabanov

Pursuant to 35 U.S.C. §202(c), it is hereby acknowledged that the U.S. Government has certain rights in the invention described herein, which was made in part with funds from the National Science

5 Foundation.

FIELD OF THE INVENTION

The present invention relates to compositions for the delivery of biological agents, 10 including, without limitation, therapeutic and diagnostic agents, and in particular to compositions comprising such agents and a complex formed of a block ionomer and an oppositely charge surfactant that exhibits combined properties of amphiphilic block 15 copolymers and polyelectrolyte-surfactant complexes.

DESCRIPTION OF RELATED ART

20 Polyelectrolyte complexes from DNA and a block ionomer containing a nonionic water soluble segment, e.g., poly(ethylene oxide) (PEO), and a polycation segment have been recently proposed to facilitate delivery of macromolecules, e.g., nucleic acids, into living cells, as a means of implementing gene therapy. In these complexes, the charges of the 25 DNA are neutralized by the polycation segments while the complex remains soluble due to the effect of the PEO segments. Such systems belong to a broader class of polyelectrolyte complexes formed by block ionomers. Published reports on complexes from PEO-*b*-poly(L- 30 lysine) cation and PEO-*b*-poly( $\alpha$ - $\beta$ -aspartate) anion, as well as PEO-*b*-polymethacrylate anion and poly(N-ethyl-4-vinylpyridinium) cation have suggested that they

represent a new type of chemical entity, with combined properties of amphiphilic block copolymers and polyelectrolyte complexes. Such systems are reported to be stable and soluble in aqueous solution and can 5 form a microphase from the neutralized polyion segments surrounded by a shell from PEO segments. In addition, these complexes appear to form micelle-like aggregates with a concentration dependence resembling those characterized by a critical micelle 10 concentration (CMC). At the same time, these systems behave like regular polyelectrolyte complexes, in that they are salt-sensitive since they tend to dissociate as the salt concentration increases beyond a critical value. Furthermore, they have been shown to 15 participate in substitution reactions involving neutralized polyion segments. These systems are produced as a result of a polyion coupling reaction after mixing polyelectrolyte components. The stability of such systems critically depends on the 20 number of the salt bonds between interacting polyelectrolytes of opposite charge. It takes at least ten salt bonds to form a cooperative system (Papisov and Litmanovich, *Adv. Polym. Sci.*, 1988, 90: 139). Preferably, the number of salt bonds should be 25 twenty or more. This means that the complexes do not form if the net charge of the molecules is less than the minimal number needed to form the required number of salt bonds, i.e., less than 10 to 20. If it is assumed that a minimal molecular mass of a charged 30 unit in the molecule of a polyelectrolyte is about 70, then the molecular mass of the components in such system should be more than about 700, preferably more than about 1,400. In practice, however, many charged units have molecular masses of about 200 and more. 35 Furthermore, many interacting molecules are weak bases or acids that at physiological pH are charged 20 to

30%. This shall put the minimal molecular mass of polyions in these systems at least 3 to 5 times higher, i.e., at least about 2,000 to 7,000.

5 Normally, the molecular masses of polynucleotides used in such systems are 8,000 to 10,000 and more. These systems therefore have a fundamental limitation in that they cannot be formed with substances having less than about 10-20 charges and molecular masses less than about 2,000-7,000.

10

## SUMMARY OF THE INVENTION

In accordance with the present invention,  
there is provided a composition of matter comprising  
a therapeutic or diagnostic agent and a supramolecular  
complex, said complex comprising as constituents (i) a  
block copolymer, having at least one nonionic, water  
soluble segment and at least one polyionic segment,  
and (ii) at least one charged surfactant having  
hydrophobic groups, the charge of said surfactant  
being opposite to the charge of the polyionic segment  
of said block copolymer the constituents of said  
complex being bound by interaction between said  
opposite charges and between surfactant hydrophobic  
groups, and with the proviso that when said  
therapeutic or diagnostic agent is an ionic substance  
having a net charge opposite to the charge of said  
block copolymer, the net charge of said therapeutic or  
diagnostic agent is no more than 10.

30 In formulating the above-mentioned supramolecular complex, the polyionic segment of the block copolymer may be polyanionic, in which case the surfactant is a cationic surfactant, or polycationic, in which case the surfactant is an anionic surfactant. The ratio of the net charge of the surfactant to the net charge of the polyionic segment present in the block copolymer of the complex is between about 0.01

35

and about 100, more preferably, between about 0.1 and about 10.

The biological agent compositions of the present invention afford many advantages over the above-mentioned, previously reported block ionomer-polyelectrolyte complexes. For example, the compositions of this invention can be used to improve the therapeutic index with relatively low-molecular mass biological agents, and biological agents having less than 10 charges. Further, they can facilitate administration of biological agents by increasing their aqueous solubility. They also increase the stability and decrease side effects of the biological agents in the body. They further increase bioavailability of the biological agent incorporated therein, after administration to the body. In addition the compositions of the invention provide for site-specific drug delivery and release in sites with acidic pH, such as tumors, bacteria, stomach, muscle tissues, or sites with alkali pH such as the gastrointestinal tract. They further provide for compartment-specific delivery of both macromolecule and small molecule biological agents into cells by releasing the biological agent in early endosomes, and enhancing its transport in the cytoplasm and cellular compartments.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphical representation of binding isotherms of cetylpyridinium bromide (1) and dodecylpyridinium bromide (2) to polyethylene oxide-block-poly(sodium methacrylate), in which  $\beta$ , a fraction of occupied binding sites, is plotted as a function of surfactant concentration.

Figure 2 is a graphical representation showing turbidity in mixtures of cetylpyridinium

- 5 -

bromide and polyethylene oxide-block-poly(sodium methacrylate) (1) and cetylpyridinium bromide and poly(sodium methacrylate) (2), as a function of the composition of the mixture.

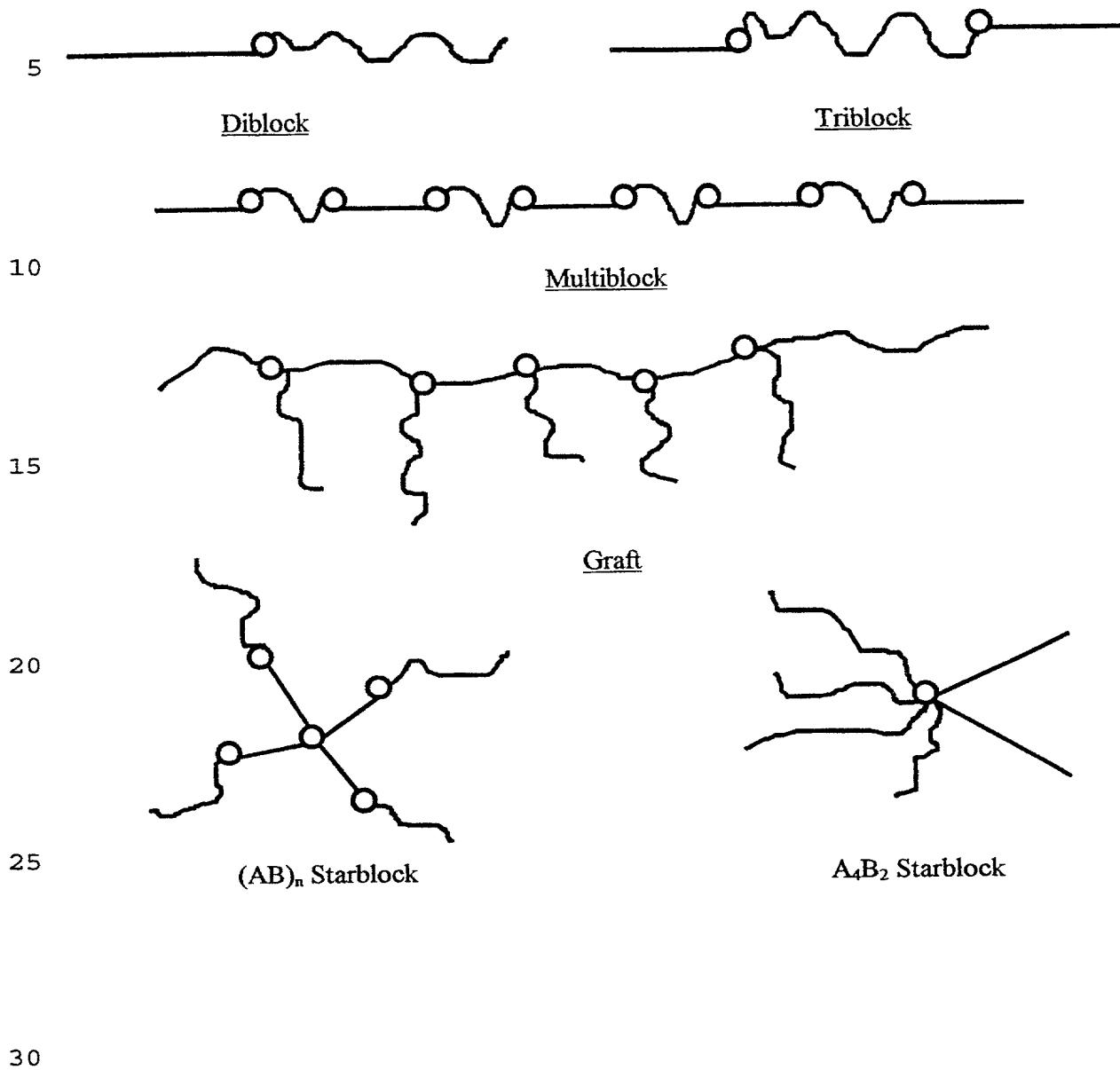
5       **Figure 3** graphically illustrates the zeta potential (Figure 4a) and effective diameter (Figure 4b) of particles formed in the mixture of cetylpyridinium bromide and poly(sodium methacrylate) at various ratios of the surfactant to the ionic units 10 of block copolymer.

15       **Figure 4** shows the dependence of the degree of conversion in the polyion coupling reaction for the mixture of cetylpyridinium bromide and polyethylene oxide-block-polymethacrylic acid.

**DETAILED DESCRIPTION OF THE INVENTION**

Filed concurrently with this application is an application entitled "COMPOSITIONS FOR DELIVERY OF BIOLOGICAL AGENTS AND METHODS FOR THE PREPARATION THEREOF" (Docket No. UNMC 63117A), with Alexander V. Kabanov, Adi Eisenberg and Victor A. Kabanov as the named inventors. The entire disclosure of Docket No. UNMC 63117A is hereby incorporated by reference herein.

25       The block copolymers used in the practice of this invention are most simply defined as conjugates of at least two different polymer segments (see, for example, Tirrel, *Interactions of Surfactants with Polymers and Proteins*. Goddard and Ananthapadmanabhan, 30 Eds., pp. 59 et seq., CRC Press (1992)). Some block copolymer architectures are presented below:



30

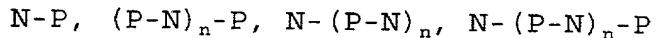
The simplest block copolymer architecture contains two segments joined at their termini to give an A-B type diblock. Consequent conjugation of more than two segments by their termini yields A-B-A type triblock, ...ABAB... type multiblock, or even

multisegment ...ABC... architectures. If a main chain in the block copolymer can be defined in which one or several repeating units are linked to different polymer segments, then the copolymer have a graft 5 architecture, e.g.  $A(B)_n$  type. More complex architectures include for example  $(AB)_n$  or  $A_nB_m$  starblocks that have more than two polymer segments linked to a single center.

One method to produce block copolymers 10 includes anionic polymerization with sequential addition of two monomers (see, for example, Schmolka, *J. Am. Oil Chem. Soc.* 1977, 54: 110; Wilczek-Vera et al., *Macromolecules* 1996, 29: 4036). This technique yields block copolymers with a narrow molecular mass 15 distribution of the polymeric segments. Solid-phase synthesis of block copolymers has been developed recently that permit controlling the growth of the polymer segments with very high precision (Vinogradov et al., *Bioconjugate Chemistry* 1996, 7: 3). In some 20 cases the block copolymers are synthesized by initiating polymerization of a polymer segment on ends of another polymer segment (Katayose and Kataoka, *Proc. Intern. Symp. Control. Rel. Bioact. Materials*, 1996, 23: 899) or by conjugation of complete polymer 25 segments (Kabanov et al., *Bioconjugate Chem.* 1995, 6: 639; Wolfert et al., *Human Gene Ther.* 1996, 7: 2123). Properties of block copolymers in relation to this invention are determined by (1) block copolymer architecture and (2) properties of the polymer 30 segments. They are independent of the chemical structure of the links used for conjugation of these segments (see, for example, Tirrel, *supra*; Sperling, *Introduction to Physical Polymer Science*, 2d edn., p. 46 et seq., John Wiley & Sons (1993)).

35 In one preferred embodiment the block copolymer is selected from the group consisting of

### polymers of formulas



5 wherein N is a nonionic, water soluble  
segment ("N-type segment"), P is polyion segment  
("P-type segment") and n is an integer from 1 to 5000.  
It is preferred that the degrees of polymerization of  
N-type and P-type segments are from about 3 to about  
10 50000, more preferably from about 5 to about 5000,  
still more preferably from about 20 to about 500. If  
more than one segment of the same type comprise one  
block copolymer, then these segments may all have the  
same lengths or may have different lengths.

1-sulfonic acid, 4-styrene sulfonic acid, trans-traumatic acid, vinylsulfonic acid, vinyl phosphonic acid, vinyl benzoic acid and vinyl glycolic acid.

Preferred polycation P-type segments include but are not limited to polyamino acid (e.g., polylysine), alkanolamine esters of polymethacrylic acid (e.g., poly-(dimethylammonioethyl methacrylate), polyamines (e.g., spermine, polyspermine, polyethyleneimine), polyvinyl pyridine, and the quaternary ammonium salts of said polycation segments.

It is preferred to use nontoxic and non-immunogenic polymer-forming N-type and P-type segments. Because of elevated toxicity and immunogenicity of cationic peptides the non-peptide P-type segments are particularly preferred.

In the case of block copolymers having at least one polyanionic segment, the nonionic segment may include, without limitation, polyetherglycols (e.g. poly(ethylene oxide), poly(propylene oxide)) copolymers of ethylene oxide and propylene oxide, polysaccharides (e.g. dextran), products of polymerization of vinyl monomers (e.g., polyacrylamide, polyacrylic esters (e.g., polyacroloyl morpholine), polymethacrylamide, poly(N-(2-hydroxypropyl) methacrylamide, polyvinyl alcohol, polyvinyl pyrrolidone, polyvinyltriazole, N-oxide of polyvinylpyridine), polyortho esters, polyamino acids, polyglycerols (e.g., poly-2-methyl-oxazoline, poly-2-ethyl oxazoline) and copolymers and derivatives thereof.

Block copolymers comprising at least one polycationic segment may be similarly formulated using nonionic segments such as polyetherglycols (e.g., polyethylene glycol) or copolymers of ethylene oxide and propylene oxide. See, for example, Bronstein et al., Proc. Am. Chem. Soc., Division of Polymeric

Materials: Science and Engineering, 76: 52 (1997); Kabanov et al., U.S. Patent 5,656,611; Spatz et al., Macromolecules, 29: 3220 (1996); Wolfert et al., Human Gene Ther., 7: 2123 (1996); Harada and Kataoka, 5 Macromolecules, 29: 3220 (1996).

The term surfactant is used herein in a most general sense to encompass any surface active agent that is adsorbed at interface (see, for example, Martin, *Physical Pharmacy*, 4th edn., p. 370 et seq., 10 Lea & Febiger, Philadelphia, London, 1993). These surface active agents in particular decrease the surface tension at the air-water interface in aqueous solutions (see, for example, Martin, *Physical Pharmacy*, 4th edn., p. 370 et seq., Lea & Febiger, 15 Philadelphia, London, 1993) and include without limitation micelle forming amphiphiles, soaps, lipids, surface active drugs and other surface active biological agents, and the like (see, for example, Martin, *Physical Pharmacy*, 4th edn., Lea & Febiger, 20 Philadelphia, London, 1993; Marcel Dekker, New York, Basel, 1979; Atwood and Florence, *J. Pharm. Pharmacol.* 1971, 23: 242S; Atwood and Florence, *J. Pharm. Sci.* 1974, 63: 988; Florence and Attwood, *Physicochemical Principles of Pharmacy*, 2nd edn., p. 180 et seq., 25 Chapman and Hall, New York, 1988; Hunter, *Introduction to Modern Colloid Science*, p. 12 et seq., Oxford University Press, Oxford, 1993). The term cationic surfactant is used herein to encompass, without limitation any surfactant that can produce cation groups in aqueous solution. This includes, without limitation strong bases (e.g., quaternary ammonium or pyridinium salts, and the like) that dissociate in aqueous solution to form cationic groups and relatively weak bases (e.g., primary amines, secondary amines, and the like) that protonate in aqueous 30 35 solution to produce a cationic group as a result of an

acidic-basic reaction. Similarly, the term anionic surfactant is used herein to encompass, without limitation any surfactant that can produce anionic groups in aqueous solution. This includes, without limitation strong acids and their salts (e.g., alkylsulfates, alkylsulfonates, alkylphosphonates, and the like) that dissociate in aqueous solution to form anionic groups and weak acids (e.g., carboxylic acids) that ionize in aqueous solution to produce an anionic group as a result of an acidic-basic reaction.

The charged surfactants that may be used in the practice of this invention are broadly characterized as cationic and anionic surfactants having hydrophobic/lipophilic groups, i.e., the groups poorly soluble in water, and/or revealing an ability to adsorb at water-air interface, and/or solubilize in organic solvents with low polarity and/or self-assemble in aqueous media to form a nonpolar microphase. The use of such compounds is an important feature of this invention. The interactions of hydrophobic groups of surfactant molecules with each other contribute to cooperative stabilization of the ionic complexes between the block copolymers and surfactants of the opposite charge in the compositions of the current invention, as will be further described below. Typically, the cationic surfactants will be lipophilic quaternary ammonium salts, lipopolyamines, lipophilic polyamino acids or a mixture thereof, particularly those proposed heretofore as a constituent of cationic lipid formulations for use in gene delivery. Various examples of classes and species of suitable cationic surfactants are provided hereinbelow.

Cationic surfactants that can be used in the compositions of the invention include, but are not limited to primary amines (e.g., hexylamine,

heptylamine, octylamine, decylamine, undecylamine,  
dodecylamine, pentadecyl amine, hexadecyl amine,  
oleylamine, stearylamine, diaminopropane,  
diaminobutane, diaminopentane, diaminohexane,  
5 diaminoheptane, diaminoctane, diaminononane,  
diaminodecane, diaminododecane), secondary amines  
(e.g., N,N-distearylamine, adrenolutin, adrenalone,  
adrenolglomerulotropin, albuterol, azacosterol,  
benzoctamine, benzydamine, carazolol, cetamolol,  
10 spirogermanium), tertiary amines (e.g.,  
N,N',N'-polyoxyethylene(10)-N-tallow-1,3-diaminopropane,  
acecainide, adiphenine hydrochloride, adinozalam,  
ahistan, alloclamide, allocryptopyne, almitrine,  
amitriptyline, anileridine, aprindine, bencyclane,  
15 benoxinate, biphenamine, brompheniramine, bucumolol,  
bufetolol, bufotenine, bufuralol, bunaftine,  
bunitrolol, bupranolol, butacaine, butamirate,  
butethamate, butofilonol, butoxycaine, butriptyline,  
captodiamine, caramiphen hydrochloride,  
20 carbetapentane, carbinoxamine, carteolol, cassaidine,  
cassaine, cassamine, chlorpromazine, dimenoxadol,  
dimethazan, diphehydramine, orphenandrine, pyrilamine,  
pyrisuccidianol, succinylcholone iodide, tetracaine,  
and the like), quaternary ammonium salts, which  
25 include aromatic and non-aromatic ring-containing  
compounds (e.g. dodecyltrimethylammonium bromide,  
hexadecyltrimethylammonium bromide,  
alkyltrimethylammonium bromide,  
tetradecyltrimethylammonium bromide, benzalkonium  
30 chloride, benzethonium chloride, benzoquinonium  
chloride, benzoxonium chloride, bibenzonium bromide,  
cetalkonium chloride, cethexonium bromide, benzylonium  
bromide, benzylidimethyldodecylammonium chloride,  
benzylidimethylhexadecylammonium chloride,  
35 benzyltrimethylammonium methoxide,  
cetyltrimethylammonium bromide,

dimethyldioctadecyl ammonium bromide (DDAB) (see, e.g., Whitt et al., *Focus*, 1991, 13: 8), methylbenzethonium chloride, decamethonium chloride, methyl mixed trialkyl ammonium chloride, methyl 5 trioctylammonium chloride, N-alkyl pyridinium salts, N-alkylpiperidinium salts, quinaldinium salts, amprolium, benzylpyrinium, bisdequalinium halides, azonium and azolium salts such as anisotropine methylbromide, butropium bromide, N- 10 butylscopolammonium bromide, tetrazolium blue, quinolinium derivatives (such as atracurium besylate), piperidinium salts, such as bevonium methyl sulfate and thiazolium salts, such as beclotiamine), 1,2-diacyl-3-(trimethylammonio)propane (acyl group = 15 dimyristoyl, dipalmitoyl, distearoyl, dioleoyl), 1,2-diacyl-3-(dimethylammonio)propane (acyl group = dimyristoyl, dipalmitoyl, distearoyl, dioleoyl), 1,2-dioleoyl-3-(4'-trimethylammonio)butanoyl-sn-glycerol, 1,2-dioleoyl-3-succinyl-sn-glycerol choline ester, cholesteryl 20 (4'-trimethylammonio) butanoate), heterocyclic amines (e.g., azacuclonol, azaperone, azatadine, benzetimide, benziperylon, benzylmorphine, bepridil, biperidene, budipine, buphanamine, buphanidine, butaperazine, 25 butorphanol, buzepide, calycanthine, carpipramine), imidazoles (e.g., azanidazole, azathiopropine, bifonazole, bizantrene, butacanazole, cafaminol), triasoles (e.g., bitertanol), tetrazoles (e.g., azosemide), phenothiazines (e.g., azures A, B, C), 30 aminoglycans (e.g., daunorubicin, doxorubicin, carminomycin, 4'-epiadiamycin, 4-demethoxy-daunomycin, 11-deoxydaunorubicin, 13-deoxydaunorubicin, adriamycin-14-benzoate, adriamycin-14-actanoate, adriamycin-14- 35 naphthaleneacetate), rhodamines (e.g. rhodamine 123), acridines (e.g. acranil, acriflavine, acrisorcin),

dicationic bolaform electrolytes (C<sub>12</sub>Me<sub>6</sub>; C<sub>12</sub>Bu<sub>6</sub>),  
dialkylglycetylphosphorylcholine, lysolecithin),  
cholesterol hemisuccinate choline ester,  
lipopolyamines (e.g., dioctadecylamidoglycylspermine  
5 (DOGS), dipalmitoyl phosphatidylethanolamidospermine  
(DPPES), N'-octadecylsperminecarboxamide  
hydroxytrifluoroacetate, N',N''-dioctadecylspermine-  
carboxamide hydroxytrifluoroacetate,  
N'-nonafluoropentadecylsperminecarboxamide  
10 hydroxytrifluoroacetate, N',N''-dioctyl  
(sperminecarbonyl)glycinamide hydroxytrifluoroacetate,  
N'-(heptadecafluorodecyl)-N'-(nonafluoropentadecyl)-  
sperminecarbonyl)glycinamide hydroxytrifluoroacetate,  
N'-[3,6,9-trioxa-7-(2'-oxaeicos-11'-enyl)heptaeicos-  
15 18-enyl]sperminecarboxamide hydroxytrifluoroacetate,  
N'-(1,2-dioleoyl-sn-glycero-3-phosphoethanoyl)spermine  
carboxamide hydroxytrifluoroacetate) (see, for  
example, Behr et. al., *Proc. Natl. Acad. Sci.* 1989,  
86: 6982; Remy et al., *Bioconjugate Chem.* 1994, 5:  
20 647), 2,3-dioleyloxy-N-[2(spermine-carboxamido)  
ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate  
(DOSPA) (see, for example, Ciccarone et al., *Focus*  
1993, 15: 80), N,N<sub>1</sub>,N<sub>11</sub>,N<sub>111</sub>-tetramethyl-N,N<sub>1</sub>,N<sub>11</sub>,N<sub>111</sub>-  
tetrapalmitylspermine (TM-TPS) (Lukow et al., *J.*  
25 *Virol.* 1993, 67:4566), N-[1-(2,3-dioleyloxy)propyl]  
-N,N,N-trimethylammonium chloride (DOTMA) (see, for  
example, Felgner, et al., *Proc. Natl. Acad. Sci. USA*  
1987, 84: 7413; Ciccarone et al., *Focus* 1993, 15: 80),  
1,2-dioleoyl-3-dimethyl-hydroxyethyl ammonium bromide  
30 (DORI) (see, for example, Felgner et al., *J. Biol.*  
*Chem.* 1994, 269:2550), 1,2-dioleyloxypropyl-  
3-dimethyl-hydroxyethyl ammonium bromide (DORIE) (see,  
for example, Felgner et al., *J. Biol. Chem.* 1994, 269:  
2550), 1,2-dioleyloxypropyl-3-dimethyl-hydroxypropyl  
35 ammonium bromide (DORIE-HP) (see, for example, Felgner  
et al., *J. Biol. Chem.* 1994, 269:2550),

1,2-dioleyloxypropyl-3-dimethyl-hydroxybutyl ammonium bromide (DORIE-HB) (see, for example, Felgner et al., *J. Biol. Chem.* 1994, 269: 2550), 1,2-dioleyloxypropyl-3-dimethyl-hydroxypentyl ammonium bromide (DORIE-HPe) (see, for example, Felgner et al., *J. Biol. Chem.* 1994, 269: 2550), 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE) (see, for example, Felgner et al., *J. Biol. Chem.* 1994, 269: 2550), 1,2-dipalmitoyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DPRIE) (see, for example, Felgner et al., *J. Biol. Chem.* 1994, 269: 2550), 1,2-distearoyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DSRIE) (see, for example, Felgner et al., *J. Biol. Chem.* 1994, 269: 2550), N,N-dimethyl-N-[2-(2-methyl-4-(1,1,3,3-tetramethylbutyl-1)-phenoxy)ethoxy]ethyl]-benzenemethanaminium chloride (DEBDA), N-[1-(2,3-dioleyloxy)propyl]-N,N,N,-trimethylammonium methylsulfate (DOTAB), lipopoly-L(or D)-lysine (see, for example, Zhou, et al., *Biochim. Biophys. Acta* 1991, 1065: 8), poly(L (or D)-lysine conjugated to N-glutarylphosphatidylethanolamine lysine (see, for example, Zhou, et al., *Biochim. Biophys. Acta* 1991, 1065: 8), didodecyl glutamate ester with pendant amino group ( $C_{12}\text{GluPhC}_n\text{N}^+$ ) (see, for example, Behr, *Bioconjugate Chem.* 1994, 5: 382), ditetradecyl glutamate ester with pendant amino group ( $C_{14}\text{GluC}_n\text{N}^+$ ) (see, for example, Behr, *Bioconjugate Chem.* 1994, 5: 382), 9-(N',N''-dioctadecylglycinamido)acridine (see, for example, Remy et al., *Bioconjugate Chem.* 1994, 5: 647), ethyl 4-[[N-[3-bis(octadecylcarbamoyl)-2-oxapropylcarbonyl]glycinamido]pyrrole-2-carboxamido]-4-pyrrole-2-carboxylate (see, for example, Remy et al., *Bioconjugate Chem.* 1994, 5: 647), N',N''-dioctadecylornithylglycinamide hydrotrifluoroacetate (see, for example, Remy et al., *Bioconjugate Chem.* 1994, 5: 647), cationic derivatives

of cholesterol (e.g., cholesteryl-3(-oxysuccinamidoethylenetrimethylammonium salt, cholesteryl-3(-oxysuccinamidoethylenedimethylamine, cholesteryl-3(-carboxyamidoethylenetrimethylammonium salt, cholesteryl-3(-carboxyamidoethylenedimethylamine, 3([N-(N',N'-dimethylaminoetane-carbomoyl] cholesterol) (see, for example, Singhal and Huang, In *Gene Therapeutics*, Wolff, Ed., p. 118 et seq., Birkhauser, Boston, 1993), pH-sensitive cationic 10 lipids (e.g., 4-(2,3-bis-palmitoyloxy-propyl)-1-methyl-1H-imidazole, 4-(2,3-bis-oleoyloxy-propyl)-1-methyl-1H-imidazole, cholesterol-(3-imidazol-1-yl propyl) carbamate, 2,3-bis-palmitoyl-propyl-pyridin-4-yl-amine) and the like (see, for example, Budker et 15 al., *Nature Biotechnology* 1996, 14: 760).

Especially useful in the context of gene delivery and other applications are compositions comprising mixtures of cationic surfactant and nonionic surfactants including, but not limited to 20 dioloyl phosphatidylethanolamine (DOPE), dioloyl phosphatidylcholine (DOPC) (see, for example, Felgner, et al., *Proc. Natl. Acad. Sci. USA* 1987; Singhal and Huang, In *Gene Therapeutics*, Wolff, Ed., p. 118 et seq., Birkhauser, Boston, 1993). This includes, in 25 particular, commercially available cationic lipid compositions including but not limited to LipofectAMINE™, Lipofectine®, DMRIE-C, CellFICTIN™, LipofectACE™, Transfectam reagents (see, for example, Ciccarone et al., *Focus* 1993, 15: 80; Lukow et al., *J. Virol.* 1993, 67: 4566; Behr, *Bioconjugate Chem.* 1994, 5: 382; Singhal and Huang, In *Gene Therapeutics*, Wolff, Ed., p. 118 et seq., Birkhauser, Boston, 1993; GIBCO-BRL Co.; Promega Co., Sigma Co) and other 30 cationic lipid compositions used for transfection of cells (see, for example, Felgner et al., *J. Biol. Chem.* 1994, 269: 2550; Budker et al., *supra*.

The anionic surfactants that can be used in the compositions of the present invention include, but are not limited to alkyl sulfates, alkyl sulfonates, fatty acid soaps, including salts of saturated and 5 unsaturated fatty acids and derivatives (e.g., adrenic acid, arachidonic acid, 5,6-dehydroarachidonic acid, 20-hydroxyarachidonic acid, 20-trifluoro arachidonic acid, docosahexaenoic acid, docosapentaenoic acid, docosatrienoic acid, eicosadienoic acid, 10 7,7-dimethyl-5,8-eicosadienoic acid, 7,7-dimethyl-5,8-eicosadienoic acid, 8,11-eicosadiynoic acid, eicosapentaenoic acid, eicosatetraynoic acid, eicosatrienoic acid, eicosatriynoic acid, eladic acid, isolinoleic acid, 15 linoelaidic acid, linoleic acid, linolenic acid, dihomo- $\gamma$ -linolenic acid,  $\gamma$ -linolenic acid, 17-octadecynoic acid, oleic acid, phytanic acid, stearidonic acid, 2-octenoic acid, octanoic acid, nonanoic acid, decanoic acid, undecanoic acid, unde 20 20 celenic acid, lauric acid, myristoleic acid, myristic acid, palmitic acid, palmitoleic acid, heptadecanoic acid, stearic acid, nonanedecanoic acid, heneicosanoic acid, docasanoic acid, tricosanoic acid, tetracosanoic acid, cis-15-tetracosenoic acid, hexacosanoic acid, 25 heptacosanoic acid, octacosanoic acid, triocantanoic acid), salts of hydroxy-, hydroperoxy-, polyhydroxy-, epoxy- fatty acids (see, for example, Ingram and Brash, *Lipids* 1988, 23:340; Honn et al., *Prostaglandins* 1992, 44: 413; Yamamoto, *Free Radic.* 30 *Biol. Med.* 1991, 10: 149; Fitzpatrick and Murphy, *Pharmacol. Rev.* 1989, 40: 229; Muller et al., *Prostaglandins* 1989, 38:635; Falgueyret et al., *FEBS Lett.* 1990, 262: 197; Cayman Chemical Co., 1994 Catalog, pp. 78-108), salts of saturated and 35 unsaturated, mono- and poly-carboxylic acids (e.g., valeric acid, *trans*-2,4-pentadienoic acid, hexanoic

acid, *trans*-2-hexenoic acid, *trans*-3-hexenoic acid, 2,6-heptadienoic acid, 6-heptenoic acid, heptanoic acid, pimelic acid, suberic acid, sebacic acid, azelaic acid, undecanedioic acid, decanedicarboxylic acid, undecanedicarboxylic acid, dodecanedicarboxylic acid, hexadecanedioic acid, docosenedioic acid, tetracosanedioic acid, agaricic acid, aleuritic acid, azafrin, bendazac, benfurodil hemisuccinate, benzylpenicillinic acid, *p*-(benzylsulfonamido)benzoic acid, biliverdine, bongrekic acid, bumadizon, caffeic acid, calcium 2-ethylbutanoate, capabenic acid, carprofen, cefodizime, cefmenoxime, cefixime, cefazadone, cefatrizine, cefamandole, cefoperazone, ceforanide, cefotaxime, cefotetan, cefonicid, 15 cefotiam, cefoxitin, cephamycins, cetiridine, cetraric acid, cetraxate, chaulmoorgic acid, chlorambucil, indomethacin, protoporphyrin IX, protizinic acid), prostanoic acid and its derivatives (e.g., prostaglandins) (see, for example, Nelson et al., *C&EN* 1982, 30-44; Frolich, *Prostaglandins*, 1984, 27: 349; Cayman Chemical Co., 1994 Catalog, pp. 26-61), leukotrienes and lipoxines (see for example, Samuelsson et al., *Science* 1987, 237: 1171; Cayman Chemical Co., 1994 Catalog, pp. 64-75), alkyl 25 phosphates, O-phosphates (e.g., benfotiamine), alkyl phosphonates, natural and synthetic lipids (e.g., dimethylallyl pyrophosphate ammonium salt, S-farnesylthioacetic acid, farnesyl pyrophosphate, 2-hydroxymyristic acid, 2-fluoropalmitic acid, inositoltrphosphates, geranyl pyrophosphate, geranygeranyl pyrophosphate,  $\alpha$ -hydroxyfarnesyl phosphonic acid, isopentyl pyrophosphate, 30 phosphatidylserines, cardiolipines, phosphatidic acid and derivatives, lysophosphatidic acids, sphingolipids and like), synthetic analogs of lipids such as 35 sodium-dialkyl sulfosuccinate (e.g., Aerosol OT<sup>®</sup>),

n-alkyl ethoxylated sulfates, n-alkyl monothiocarbonates, alkyl- and arylsulfates (asaprol, azosulfamide, p-(benzylsulfonamido)benzoic acid, cefonicid, CHAPS), mono- and dialkyl dithiophosphates, 5 N-alkanoyl-N-methylglucamine, perfluoroalcanoate, cholate and desoxycholate salts of bile acids, 4-chloroindoleacetic acid, cucurbic acid, jasmonic acid, 7-epi jasmonic acid, 12-oxo phytodienoic acid, traumatic acid, tuberonic acid, abscisic acid, 10 acitertin, and the like.

The preferred cationic and anionic surfactants of this invention also include fluorocarbon and mixed fluorocarbon-hydrocarbon surfactants. See, for example, Mukerjee, *P. Coll. Surfaces A: Physicochem. Engin. Asp.* 1994, 84: 1; Guo et al., *J. Phys. Chem.* 1991, 95: 1829; Guo et al., *J. Phys. Chem.*, 1992, 96: 10068. The list of such surfactants that are useful in the present invention includes, but is not limited to the salts of 15 perfluoromonocarboxylic acids (e.g., pentafluoropropionic acid, heptafluorobutyric acid, nonanfluoropentanoic acid, tridecafluoroheptanoic acid, pentadecafluorooctanoic acid, heptadecafluorononanoic acid, nonadecafluorodecanoic acid, 20 perfluorododecanoic acid, perfluoropolycarboxylic acids, perfluorotetradecanoic acid) and the salts of perfluoro-polycarboxylic acids (e.g., hexafluoroglutaric acid, perfluoroadipic acid, perfluorosuberic acid, perfluorosebacic acid), double 25 tail hybrid surfactants,  $(C_mF_{2m+1})(C_nH_{2n+1})CH-OSO_3Na$  (see, for example, Guo et al., *J. Phys. Chem.*, 1992, 96: 6738, Guo et al., *J. Phys. Chem.* 1992, 96: 10068; Guo et al., *J. Phys. Chem.*, 1992, 96: 10068), 30 fluoroaliphatic phosphonates, fluoroaliphatic sulphates, and the like.

The biological agent compositions of this

invention may additionally contain nonionic or zwitterionic surfactants including but not limited to phospholipids (e.g. phosphatidylethanolamines, phosphatidylglycerols, phosphatidylinositols, diacyl 5 phosphatidylcholines, di-O-alkyl phosphatidylcholines, platelet-activating factors, PAF agonists and PAF antagonists, lysophosphatidylcholines, lysophosphatidylethanol-amines, lysophosphatidylglycerols, lysophosphatidylinositols, 10 lyso-platelet-activating factors and analogs, and the like), saturated and unsaturated fatty acid derivatives (e.g., ethyl esters, propyl esters, cholesteryl esters, coenzyme A esters, nitrophenyl esters, naphtyl esters, monoglycerids, diglycerids, 15 and triglycerids, fatty alcohols, fatty alcohol acetates, and the like), lipopolysaccharides, glyco- and sphingolipids (e.g. ceramides, cerebrosides, galactosyldiglycerids, gangliosides, lactocerebrosides, lysosulfatides, psychosines, 20 sphingomyelins, sphingosines, sulfatides), chromophoric lipids (neutral lipids, phospholipids, cerebrosides, sphingomyelins), cholesterol and cholesterol derivatives, Amphotericin B, abamectin, acediasulfone, n-alkylphenyl polyoxyethylene ether, 25 n-alkyl polyoxyethylene ethers (e.g., Triton™), sorbitan esters (e.g. Span™), polyglycol ether surfactants (Tergitol™), polyoxyethylenesorbitan (e.g., Tween™), polysorbates, polyoxyethylated glycol monoethers (e.g., Brij™, polyoxylethylene 9 lauryl 30 ether, polyoxylethylene 10 ether, polyoxylethylene 10 tridecyl ether), lubrol, copolymers of ethylene oxide and propylene oxide (e.g., Pluronic™, Pluronic R™, Teronic™, Pluradot™), alkyl aryl polyether alcohol (Tyloxapol™), perfluoroalkyl polyoxylated amides, 35 N,N-bis[3-D-gluconamidopropyl]cholamide, decanoyl-N-methylglucamide, n-decyl

$\alpha$ -D-glucopyranoside, n-decyl  $\beta$ -D-glucopyranoside,  
n-decyl  $\beta$ -D-maltopyranoside, n-dodecyl  
 $\beta$ -D-glucopyranoside, n-undecyl  $\beta$ -D-glucopyranoside,  
n-heptyl (-D-glucopyranoside, n-heptyl  
5  $\beta$ -D-thioglucopyranoside, n-hexyl  $\beta$ -D-glucopyranoside,  
n-nonanoyl  $\beta$ -D-glucopyranoside  
1-monooleyl-rac-glycerol, nonanoyl-N-methylglucamide,  
n-dodecyl  $\alpha$ -D-maltoside, n-dodecyl  $\beta$ -D-maltoside,  
N,N-bis[3-gluconamidepropyl]deoxycholamide, diethylene  
10 glycol monopentyl ether, digitonin,  
heptanoyl-N-methylglucamide, heptanoyl-N-  
methylglucamide, octanoyl-N-methylglucamide, n-octyl  
 $\beta$ -D-glucopyranoside, n-octyl  $\alpha$ -D-glucopyranoside,  
n-octyl  $\beta$ -D-thiogalactopyranoside, n-octyl  
15  $\beta$ -D-thioglucopyranoside, betaine ( $R_1R_2R_3N^+R'CO_2^-$ , where  
 $R_1R_2R_3R'$  hydrocarbon chains), sulfobetaine  
( $R_1R_2R_3N^+R'SO_3^-$ ), phospholipids (e.g. dialkyl  
phosphatidylcholine), 3-[(3-cholamidopropyl)-  
dimethylammonio]-2-hydroxy-1-propanesulfonate,  
20 3-[(3-cholamidopropyl)-dimethylammonio]-1-  
propanesulfonate, N-decyl-N,N-dimethyl-3-  
ammonio-1-propanesulfonate, N-dodecyl-N,N-dimethyl-  
3-ammonio-1-propanesulfonate, N-hexadecyl-N,N-  
dimethyl-3-ammonio-1-propanesulfonate,  
25 N-octadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate,  
N-octyl-N,N-dimethyl-3-ammonio-1-propanesulfonate,  
N-tetradecyl-N,N-dimethyl-3-ammonio-1-  
propanesulfonate, dialkyl phosphatidylethanolamine.

The biological agents that may be used in  
30 the practice of this invention include those having  
utility in diagnostics or imaging, as well as those  
capable of acting on a cell, organ or organism to  
create a change in the functioning of the cell, organ  
or organism, including but not limited to  
35 pharmaceutical agents. Such biological agents include  
a wide variety of substances that are used in

diagnostics, therapy, immunization or otherwise are applied to combat human and animal disease. Such agents include but are not limited to analgesic agents, anti-inflammatory agents, antibacterial agents, 5 antiviral agents, antifungal agents, antiparasitic agents, tumoricidal or anti-cancer agents, proteins, toxins, enzymes, hormones, neurotransmitters, glycoproteins, immunoglobulins, immunomodulators, dyes, radiolabels, radio-opaque compounds, fluorescent 10 compounds, polysaccharides, cell receptor binding molecules, anti-inflammatories, anti-glaucomic agents, mydriatic compounds and local anesthetics.

The biological agent compositions of this invention may comprise polyions, e.g., either 15 polycations, if they have more than about ten negative charges, or polyanions, if they have more than about ten negative charges. Examples of polycations include cationic polypeptides, examples of polyanions include polynucleotides. It is important, however, to note 20 that there is a fundamental limitation to the charge sign of the polyionic biological agents used in the composition of this invention with block copolymers and surfactants. The polyanionic biological agent must not (1) have the opposite charge compared to the 25 charge sign of the P-type segment of the block copolymer, and (2) have the same charge sign compared to the charge sign of the surfactant. That is if the P-type segment of the block copolymer is a polyanion and surfactant is cationic, then the biological agent must 30 not be a polycation. Similarly, if the P-type segment of the block copolymer is a polycation and surfactant is anionic, then the biological agent must not be a polyanion. The reason for such limitation is the necessity to avoid competitive binding of the 35 polyion biological agent and surfactant of the same charge to the P-type segment of the block copolymer.

Normally, the polyanionic biological agent would have comparable or higher affinity to the oppositely charged P-type segment of the block copolymer when compared to the surfactant. The competition between 5 the polyanion biological agent and the surfactant of the same charge for the electrostatic binding with the P-type segment of the opposite charge would disturb the electrostatic interactions between the surfactant and the block copolymer and result in the destruction of 10 the compositions of the current invention. For example, if polylysine having 10 repeating units is added to the complex formed between polyethylene oxide-block-poly(sodium methacrylate) block copolymer and cetylpyridinium bromide, then the substitution 15 reaction takes places resulting in the disintegration of the complex between the block copolymer and surfactant. Similarly, if a 10-mer oligonucleotide is added to the complex formed between polyethylene oxide-block-polyethyleneimine block copolymer and 20 Aerosol OT, then this complex disintegrates as a result of the substitution reaction.

The biological agents which may be used in the compositions of the invention may include, but are not limited to non-steroidal anti-inflammatories, such 25 as indomethacin, salicylic acid acetate, ibuprofen, sulindac, piroxicam, and naproxen, antiglaucomic agents such as timolol or pilocarpine, neurotransmitters such as acetylcholine, anesthetics such as dibucaine, neuroleptics such as the 30 phenothiazines (e.g., compazine, thorazine, promazine, chlorpromazine, acepromazine, aminopromazine, perazine, prochlorperazine, trifluoperazine, and thioproperazine), rauwolfa alkaloids (e.g., reserpine and deserpine), thioxanthenes (e.g., chlorprothixene 35 and tiotixene), butyrophenones (e.g., haloperidol, moperone, trifluoperidol, timiperone, and droperidol),

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diphenylbutylpiperidines (e.g., pimozde), and benzamides (e.g., sulpiride and tiapride); tranquilizers such as glycerol derivatives (e.g., mephenesin and methocarbamol), propanediols (e.g., 5 meprobamate), diphenylmethane derivatives (e.g., orphenadrine, benzotrapine, and hydroxyzine), and benzodiazepines (e.g., chlordiazepoxide and diazepam); hypnotics (e.g., zolpdem and butoxtamide); beta-blockers (e.g., propranolol, acebutonol, 10 metoprolol, and pindolol); antidepressants such as dibenzazepines (e.g., imipramine), dibenzocycloheptenes (e.g., amtriptiline), and the tetracyclics (e.g., mianserine); MAO inhibitors (e.g., phenelzine, iproniazid, and selegeline); 15 psychostimulants such as phenylethylamine derivatives (e.g., amphetamines, dexamphetamines, fenproporex, phentermine, amfepramone, and pemoline) and dimethylaminoethanols (e.g., clofenciclan, cyprodenate, aminorex, and mazindol); GABA-mimetics (e.g., progabide); alkaloids (e.g., codergocrine, 20 dihydroergocristine, and vincamine); anti-Parkinsonism agents (e.g., L-dopamine and selegeline); agents utilized in the treatment of Alzheimer's disease, cholinergics (e.g., citicoline and physostigmine); 25 vasodilators (e.g., pentoxifyline); and cerebro active agents (e.g., pyritinol and meclofenoxate).

Anti-neoplastic agents can also be used advantageously as biological agents in the compositions of the invention. Representative examples include, but are not limited to paclitaxel, 30 daunorubicin, doxorubicin, carminomycin, 4'-epiadriamycin, 4-demethoxy-daunomycin, 11-deoxydaunorubicin, 13-deoxydaunorubicin, adriamycin-14-benzoate, adriamycin-14-actanoate, 35 adriamycin-14-naphthaleneacetate, vinblastine, vincristine, mitomycin C, N-methyl mitomycin C,

bleomycin A<sub>2</sub>, dideazatetrahydrofolic acid, aminopterin, methotrexate, cholchicine and cisplatin.

Representative antibacterial agents are the aminoglycosides including gentamicin. Representative 5 antiviral compounds are rifampicin, 3'-azido-3'-deoxythymidine (AZT), and acylovir.

Representative antifungal agents are the azoles, including fluconazole, macrolides such as amphotericin B, and candididin. Representative anti-parastic 10 compounds are the antimonials. Suitable biological agents also include, without limitation vinca alkaloids, such as vincristine and vinblastine, mitomycin-type antibiotics, such as mitomycin C and N-methyl mitomycin, bleomycin-type antibiotics such as 15 bleomycin A<sub>2</sub>, antifolates such as methotrexate, aminopterin, and dideaza-tetrahydrofolic acid, taxanes, anthracycline antibiotics and others.

The compositions also can utilize a variety of 20 polypeptides, such as antibodies, toxins, such as diphtheria toxin, peptide hormones, such as colony stimulating factor, and tumor necrosis factors, neuropeptides, growth hormone, erythropoietin, and thyroid hormone, lipoproteins such as  $\mu$ -lipoprotein, proteoglycans such as hyaluronic acid, glycoproteins 25 such as gonadotropin hormone, immunomodulators or cytokines such as the interferons or interleukins, as well as hormone receptors such as the estrogen receptor.

The compositions also can comprise enzyme 30 inhibiting agents such as reverse transcriptase inhibitors, protease inhibitors, angiotensin converting enzymes, 5 $\mu$ -reductase, and the like. Typical of these agents are peptide and nonpeptide structures such as finasteride, quinapril, ramipril, 35 lisinopril, saquinavir, ritonavir, indinavir, nelfinavir, zidovudine, zalcitabine,

allophenylnorstatine, kynostatin, delaviridine, bis-tetrahydrofuran ligands (see, for example Ghosh et al., *J. Med. Chem.* 1996, 39: 3278), and didanosine. Such agents can be administered alone or in combination 5 therapy; e.g., a combination therapy utilizing saquinavir, zalcitabine, and didanosine, zalcitabine, and zidovudine. See, for example, Collier et al., *Antiviral Res.* 1996, 29: 99.

The biological agent compositions can also 10 comprise nucleotides, such as thymine, nucleic acids, such as DNA or RNA, or synthetic oligonucleotides, which may be derivatized by covalently modifying the 5' or the 3' end of the polynucleic acid molecule with hydrophobic substituents to facilitate entry into 15 cells (see for example, Kabanov et al., *FEBS Lett.* 1990, 259, 327; Kabanov and Alakhov, *J. Contr. Rel.* 1990, 28: 15). Additionally, the phosphate backbone of the polynucleotides may be modified to remove the negative charge (see, for example, Agris et al., 20 *Biochemistry* 1968, 25:6268, Cazenave and Helene in *Antisense Nucleic Acids and Proteins: Fundamentals and Applications*, Mol and Van der Krol, Eds., p. 47 et seq., Marcel Dekker, New York, 1991), or the purine or pyrimidine bases may be modified, for example, to 25 incorporate photo-induced crosslinking groups, alkylating groups, organometallic groups, intercalating groups, biotin, fluorescent and radioactive groups (see, for example, *Antisense Nucleic Acids and Proteins: Fundamentals and Applications*, Mol and Van der Krol, Eds., p. 47 et seq., Marcel Dekker, New York, 1991; Milligan et al., 30 *In Gene Therapy for Neoplastic Diseases*, Huber and Laso, Eds. P. 228 et seq., New York Academy of Sciences, New York, 1994). Such nucleic acid 35 molecules can be among other things antisense nucleic acid molecules, phosphodiester, oligonucleotide

5            $\alpha$ -anomers, ethylphosphotriester analogs, phosphorothioates, phosphorodithioates, phosphoroethyl esters, methylphosphonates, and the like (see, e.g., Crooke, *Anti-Cancer Drug Design* 1991, 6: 609; De Mesmaeker et al, *Acc. Chem. Res.* 1995, 28: 366). The compositions of the invention may also include antigen, ribozyme and aptamer nucleic acid drugs (see, for example, Stull and Szoka, *Pharm. Res.* 1995, 12: 465).

10           Included among the suitable biological agents are viral genomes and viruses (including the lipid and protein coat). Thus, formulation of the above-described compositions to include a variety of viral vectors, including complete viruses of their 15 parts, for use in gene delivery (e.g. retroviruses, adenoviruses, herpes-virus, Pox-virus) is contemplated to be within the scope of this invention. See, for example, Hodgson, *Biotechnology*, 1995, 13: 222.

20           Other suitable biological agents include oxygen transporters (e.g. porphines, porphirines and their complexes with metal ions), coenzymes and vitamins (e.g. NAD/NADH, vitamins B12, chlorophylls), and the like.

25           Suitable biological agents further include those used in diagnostics visualization methods, such as magnetic resonance imaging (e.g., gadolinium (III) diethylenetriamine pentaacetic acid), and may be a chelating group (e.g., diethylenetriamine pentaacetic acid, triethylenetriamine pentaacetic acid, ethylenediamine-tetraacetic acid, 1,2-diaminocyclo-hexane-N,N,N',N'-tetraacetic acid, N,N'-di(2-hydroxybenzyl) ethylene diamine), N-(2-hydroxyethyl) ethylene diamine triacetic acid and the like). Such biological agent may further include 30 an alpha-, beta-, or gamma-emitting radionuclide (e.g., gallium 67, indium 111, technetium 99).

Iodine-containing radiopaque molecules are also suitable diagnostic agents. The diagnostic agent may also include a paramagnetic or superparamagnetic element, or combination of paramagnetic element and 5 radionuclide. The paramagnetic elements include but are not limited to gadolinium (III), dysporsium (III), holmium (III), europium (III) iron (III) or manganese (II).

10 The composition may further include a targeting group including but not limited to antibody, fragment of an antibody, protein ligand, polysaccharide, polynucleotide, polypeptide, low molecular mass organic molecule and the like. Such targeting group can be linked covalently to the block 15 copolymer or surfactant, or can be non-covalently incorporated in the compositions through hydrophobic, electrostatic interactions or hydrogen bonds.

20 While not wishing to be bound by any particular theory, it is believed that the above- described surfactants and block copolymers of opposite charge form stable complexes due to cooperative 25 binding. (See, e.g., Goddard, In *Interactions of Surfactants with Polymers and Proteins*. Goddard and Ananthapadmanabhan, Eds., pp. 171 et seq., CRC Press, Boca Raton, Ann Arbor, London, Tokyo, 1992).

30 Cooperative binding occurs in the sense that binding of surfactant molecules to the block copolymer is enhanced by the presence of other molecules of the same or a different surfactant already bound to the same copolymer. According to the cooperative binding mechanism which is believed to underlie this invention, surfactant binds electrostatically to the oppositely charged P-type segments of the block copolymer to form supramolecular complexes. These 35 complexes are cooperatively stabilized by the interactions of the hydrophobic parts of surfactant

molecules bound to the same P-type segment with each other. Indeed, it appears that without these hydrophobic interactions, formation of the desired complex would not occur. Figure 1 presents the 5 binding isotherms for interaction of cetylpyridinium bromide and dodecylpyridinium bromide with the polyethylene oxide-block-poly(sodium methacrylate) block copolymer. As can be seen in Figure 1, a decrease in the length of the hydrophobic substituent 10 on the surfactant, from more hydrophobic cetyl- to less hydrophobic dodecyl-, decreases the stability of the complex more than ten-fold. This binding mechanism is, in particular, characteristic for surface active biological agents. See, for example, 15 Florence and Attwood, *Physicochemical Principles of Pharmacy*, 2nd edn., p.180 et seq., Chapman and Hall, New York, 1988).

Formation of the electrostatic complexes between surfactants and P-type segments results in 20 charge neutralization. As a result hydrophobicity of the complexed segments increases and aqueous solubility decreases. However, the preferred biological agent compositions remain in the aqueous solution due to the presence of the N-type segments 25 linked to the P-type segments. By varying the relative lengths and amounts of the N-type and P-type blocks it is possible to vary the hydrophilic/lipophilic properties of the complexes formed between the surfactant and block copolymer and 30 to optimize the solubility of the preferred compositions. It is preferred that the compositions of the present invention are water soluble at physiological conditions (pH, osmolarity, etc.) and temperatures. Figure 2 shows that binding of 35 cetylpyridinium bromide to poly(sodium methacrylate) segment alone results in formation of a

- 30 -

water-insoluble complex, whereas binding of the same surfactant to the block copolymer polyethylene oxide-block-poly(sodium methacrylate) yields a water-soluble complex.

5        The compositions of the present invention normally form complexes of small size that are thermodynamically stable and do not aggregate after storing in solutions for a period of time on the order of weeks or months, depending on the type of polymer  
10      present therein. The ability to produce particles of such limited size is important because small particles can easily penetrate into tissues through even small capillaries and enter cells via endocytosis. The preferred size of these particles is less than 500 nm,  
15      more preferred less than 200 nm, still more preferred less than 100 nm. These systems can be lyophilized and stored as a lyophilized powder and then re-dissolved to form solutions with the particles of the same size.

20      It is useful to consider the constituents that make up the complexes of this invention in terms of a parameter referred to herein as charge ratio of the complex. The charge ratio of the complex, herein designated as " $\phi$ ", is the ratio of the net charge of the surfactant molecules bound with one block  
25      copolymer molecule to the net charge of the P-type segments in this block copolymer. By way of example, if the surfactant molecule has two positively charged groups and five negatively charged groups, it has a "net charge" of -3. Thus, if a surfactant with the  
30      net charge  $z_1$  binds to the oppositely charged P-type segments of the block copolymer, then the charge ratio is expressed as follows:

35

$$\phi = \frac{n \cdot z_1}{Y \cdot z_2 \cdot PD} \quad (1)$$

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where  $n$  is the number of surfactant molecules bound to one block copolymer molecule,  $z_2$  is the net charge of the repeating unit of the P-type segment, PD is the degree of polymerization of the P-type segment, and  $\gamma$  is the number of P-type segments in the block copolymer molecule.

If  $\phi < 1$ , then the complex has the same charge sign as the P-type segments; if  $\phi > 1$ , the complex has the same charge sign as the surfactant; and if  $\phi = 1$ , the complex is electro-neutral.

Therefore, by varying the molar ratio of the components in the compositions the charge of the particles formed can be changed from negative to positive and vice versa. See for example Figure 3.

As a result various biological agents can be incorporated into such particles through combination of electrostatic and hydrophobic interactions. For example, the compositions from cationic biological agents (e.g., cholesteryl (4'-trimethyl ammino) butanoate and anionic block copolymer (e.g., polyethylene oxide-block-poly(sodium methacrylate)) at  $\phi > 1$  are positively charged and incorporate negatively charged polynucleotide molecules through electrostatic interactions. The compositions from anionic biological agents (e.g., Aerosol<sup>®</sup> OT) and cationic copolymer (e.g., polyethyleneimine and polyethylene oxide) at  $\phi > 1$  are negatively charged and incorporate positively charged polypeptide molecules through electrostatic interactions.

As previously noted, in the case of a composition including an anionic block copolymer and a positively charged therapeutic or diagnostic, the net charge of the latter should be no more than about 10, and preferably about 5. Likewise, in the case of a composition including a cationic block copolymer, and a negatively charged therapeutic or diagnostic agent,

the net charge of the latter should be no more than about 10, and preferably about 5.

It is further useful to introduce the parameter characterizing the ratio of the net charges of surfactant to the net charges of the P-type segments of the block copolymer in the compositions of this invention. This parameter referred herein as composition of the mixture, and herein designated "Z" is expressed as follows:

10

$$Z = \frac{z_1 \cdot C_1}{\forall z_2 \cdot PD \cdot C_2} \quad (2)$$

where  $z_1$ ,  $z_2$ ,  $\gamma$ , and PD have the same meaning as in equation (1) and  $C_1$  and  $C_2$  are the molar concentrations of the surfactant and block copolymer in the mixture.

15 While not wishing to be bound to any particulate theory, it is believed that the relationship between the charge ratio of the complex and composition of the mixture is as follows,  $\phi \geq Z$ . This means that  $n \geq C_1 C_2$ , i.e., the number of surfactant molecules bound to one block copolymer molecule is not less than the ratio of the molar concentrations of the surfactant and copolymer in the mixture. Further, when  $Z$  is less than 1, preferably less than 0.5,  $n > C_1 C_2$ , i.e., the number of surfactant molecules bound to one block copolymer molecule is more than the ratio of the molar concentrations of the surfactant and block copolymer in the mixture. The reason for the behavior expressed by those relationships is that the binding of surfactant molecules to the P-type segments of the block copolymer is enhanced by the presence of the molecules of surfactant already bound to the same block copolymer. Therefore, those relationships

reflect the cooperative binding mechanism between the surfactant and block copolymers of the current invention.

Under certain conditions the particles of the complex spontaneously form vesicles that contain an internal volume defined by the constituents of the complex. Various biologically active agents (for example, 3'-azido-3'-deoxythymidine, water soluble protease inhibitors, interleukins, insulin and the like) can be physically entrapped into the internal aqueous volume of such vesicles. The optimal conditions for the vesicle formation is determined by the composition of the mixture, so that the vesicles form when Z is more than about 0.1 and less than 100, preferably more than 0.4 and less than 20, still more preferably more than 0.7 less than 10.

Furthermore, the compositions of present invention can solubilize hydrophobic biological agents (e.g., paclitaxel) through nonpolar interactions in the microphase formed by the hydrophobic groups of the surfactant.

One important aspect of the current invention is that electrostatic interactions between the charged groups of the biologically active surface active agent and repeating units of the P-type segments of the block copolymer are pH-dependent. For example, Figure 4 shows the pH-dependency of the reaction between cetylpyridinium bromide and the copolymer of polymethacrylic acid and polyethylene oxide. It is preferred that either the P-type segment of the block copolymer or the surfactant or both the P-type segment and the surfactant represent a weak acid or weak base. The preferred compositions formed are pH-dependent in the pH-range of from about pH 2.0 to about pH 10.0, more preferably in the pH-range of from about pH 3.0 to about pH 9.0, still more

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preferably in the pH-range of from about pH 4.0 to about pH 8.0. Dissociation of the biologically active surfactant and block copolymer complexes as a result of the pH change is characterized by step-type 5 behavior, with more cooperative systems revealing sharper pH dependencies. Preferably these complexes dissociate when the pH change is about 3.0 units of pH, more preferably about 2.0 units of pH, still more preferably about 1.0 unit of pH. Since the 10 biologically active surfactant and block copolymer are linked to each other through non-covalent interactions, dissociation of the complex results in the release of the biological agent. The complexes between the surfactants and block copolymers are 15 stable in the pH range of from about pH 5.5 to about pH 8.5, preferably in the pH range of from about pH 6.5 to about pH 7.5. These complexes, however, easily dissociate when the pH shift occurs in the target tissue or cell, or other target site, which enables 20 site-specific release of the biological agents. To facilitate for the release of the surfactant active biological agent from the compositions of the first embodiment the total amount of net charge of these biological agents is no more than about 5, more 25 preferably no more than 4, still more preferably no more than 3.

The compositions of the present invention allow diverse routes of administration, including but not limited by parenteral (such as intramuscular, 30 subcutaneous, intraperitoneal, and intravenous), oral, topical, otic, topical, vaginal, pulmonary, and ocular. These compositions can take the form of tablets, capsules, lozenges, troches, powders, gels, syrups, elixirs, aqueous solutions, suspensions, 35 micelles, emulsions and microemulsions.

Conventional pharmaceutical formulations are

employed. In the case of tablets, for example, well-known carriers such as lactose, sodium citrate, and salts of phosphoric acid can be used. Disintegrants such as starch, and lubricating agents such as magnesium stearate, sodium lauryl sulfate and talc, as are commonly used in tablets, can be present. Capsules for oral administration can include diluents such as lactose and high molecular weight polyethylene glycols. When aqueous suspensions are required for oral use, the conjugate can be combined with emulsifying and suspending agents. For parenteral administration, sterile solutions of the conjugate are usually prepared, and the pH of the solutions are suitably adjusted and buffered. For intravenous use, the total concentration of solutes should be controlled to render the preparation isotonic. For ocular administration, ointments or droppable liquids may be delivered by well-known ocular delivery systems such as applicators or eyedroppers. Such compositions can include mucomimetics such as hyaluronic acid, chondroitin sulfate, hydroxypropyl methylcellulose or polyvinyl alcohol, preservatives such as sorbic acid, EDTA or benzylchronium chloride, and the usual quantities of diluents and/or carriers. For pulmonary administration, diluents and/or carriers will be selected to be appropriate to allow for formation of an aerosol.

The following examples are provided to describe the invention in further detail. These examples are intended merely to illustrate specific embodiments of the present invention and should in no way be construed as limiting the invention.

EXAMPLE 1

A. Block copolymer of tert-butyl methacrylate and ethylene oxide used in this study was prepared by sequential anionic polymerization 5 generally following the previously published procedure (Wang et al., *J. Polym. Sci., Part A: Polym. Chem.* 1992, 30: 2251). The block lengths in copolymer were 176 for the PEO and 186 for tert-butyl methacrylate segments respectively. This copolymer was hydrolyzed 10 to obtain the polyethylene oxide-block-polymethacrylic acid as described by Wang et al. (*J. Polym. Sci., Part A: Polym. Chem.* 1992, 30: 2251). The polyethylene oxide-block-poly(sodium methacrylate) was prepared by redissolving the acid form of the copolymer in 15 tetrahydrofuran : methanol mixture (95:5 v/v) and adding NaOH in methanol. The precipitate containing polyethylene oxide-block-poly(sodium methacrylate) was filtered and washed with methanol, then redissolved in water and freeze dried. The concentration of 20 carboxylate groups in the copolymer sample was estimated by potentiometric titration.

B. The binding isotherms of cetylpyridinium bromide (Sigma Co.) and dodecylpyridinium bromide (Sigma Co.) with the 25 polyethylene oxide-block-poly(sodium methacrylate) copolymer were determined potentiometrically by using the following ion-selective electrode: Ag/AgCl | 1 M NH<sub>4</sub>NO<sub>3</sub> agar bridge | reference solution (surfactant, 2.5·10<sup>-4</sup> M) | PVC membrane | sample solution | 1 M 30 NH<sub>4</sub>NO<sub>3</sub> agar bridge | Ag/AgCl. The preparation of PVC ion-selective electrode is described elsewhere (Shirahama et al., *Bull. Chem. Soc. Jpn.* 1981, 54: 375; Shirahama and Tashiro, *Bull. Chem. Soc. Jpn.* 1984, 57: 377; Mel'nikov et al., *J. Am. Chem. Soc.* 35 1995, 117: 9951). The electromotive force (emf) of the cell was measured with a digital Radiometer pHM-83

pH-meter. The electrode exhibited a good Nernstian response over the range of surfactant concentrations examined. Binding isotherms were obtained by titration of  $5 \cdot 10^{-4}$  base-mole/L polyethylene oxide-block-poly(sodium methacrylate) solutions with the solution of the corresponding surfactant. The results were corrected for the decrease in the copolymer concentration as a result of the addition of the surfactant solution. The fraction of occupied binding sites  $\beta$  was defined as follows

$$\beta = (C_t - C_f) / C_i \quad (3)$$

where  $C_t$  is the total concentration of added surfactant,  $C_f$  is the concentration of the free surfactant,  $C_f$  determined using the calibration curves (i.e., linear plots "emf vs.  $\log C_t$ " determined in the absence of polymer and  $C_t < \text{CMC}$ ), and  $C_i$  is the concentration of ionic groups of the block copolymer. The results obtained for binding of cetylpyridinium bromide and dodecylpyridinium bromide are presented in Figure 1.

#### EXAMPLE 2

Polyethylene oxide-block-poly(sodium methacrylate) was the same as described in Example 4. The homopolymer polymethacrylic acid with a  $P_w=930$  was obtained by radical polymerization (Lipatov, and Zubov, Vysokomol. Soedin., Ser. A. 1959, 1:88). Complexes formed between these polymers and cetylpyridinium bromide (Sigma Co.) were studied using turbidity measurements. At base molar concentrations of anionic polymers  $1.08 \cdot 10^{-3}$  base-mole/L; temperature  $25^\circ\text{C}$ , and pH 9.2. The turbidity measurements were carried out using a Shimadzu UV160 spectrophotometer at 420 nm after equilibration of the system typically for 3 minutes. The data are reported in Figure 2 as

(100-T) / 100, where T is transmission (%) for complexes from polyethylene oxide-block-poly(sodium methacrylate) and poly(sodium methacrylate).

5

EXAMPLE 3

The complexes between N-cetylpyridinium bromide and polyethylene oxide-block-poly(sodium methacrylate) were prepared in  $8 \cdot 10^{-4}$  base-mole/L solution of the block copolymer at 25°C and pH 9.2.

10 The  $C_t/C_i$  was varied from 0.01 to 5, where  $C_t$  is the total concentration of added surfactant, and  $C_i$  is the concentration of ionic groups of the block copolymer. Electrophoretic mobility (EPM) measurements were performed at 25°C with an electric field strength of

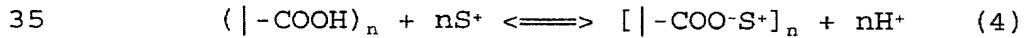
15 15-18 V/cm by using "ZetaPlus" Zeta Potential Analyzer (Brookhaven Instrument Co.) with 15 mV solid state laser operated at a laser wavelength of 635 nm. The zeta-potential of the particles was calculated from the EPM values using the Smoluchowski equation.

20 Effective hydrodynamic diameter was measured by photon correlation spectroscopy using the same instrument equipped with the Multi Angle Option. All solutions were prepared using double distilled water and were filtered repeatedly through the Millipore membrane

25 with pore size 0.22  $\mu$ M. The sizing measurements were performed at 25°C at an angle of 90°. The results are presented in Figure 3.

EXAMPLE 4

30 The interaction between a cationic surfactant ( $S^+$ ) and a weak polyacid represents an ion exchange reaction resulting in the release of the protons in accordance with the following scheme:



The equilibrium of this reaction for the mixtures of cetylpyridinium bromide with polyethylene oxide-block-poly methacrylic acid was studied at different pH by potentiometric titration (see, for example, Kabanov, Polymer Science 1994, 36:143).  
5 Polyethylene oxide-block-poly methacrylic acid was synthesized as described in Example 1. The alkali titration curves were obtained for the mixtures of N-cetylpyridinium bromide (Sigma Co.) with polyethylene  
10 oxide-block-poly methacrylic acid. The total concentration of the surfactant was equal to the concentration of the ionizable groups of the polyacid. The degree of conversion,  $\theta$ , in the ion exchange reaction (5) was determined from original titration  
15 curves on the assumption that all alkali is consumed for neutralization of protons released as a result of this reaction. For a weak polyacid  $\theta$  at a given pH is expressed as follows

20

$$\theta = (m_b/V + [H^+] - \sqrt{K_a C_o})/C_o, \quad (5)$$

where  $M_b$  is the number of moles of the added base,  $V$  is  
25 the current volume of the reaction system,  $K_a$  is the characteristic dissociation constant,  $C_o$  is the base-molar concentration of the polyacid. The results are presented in Figure 4.

30

#### EXAMPLE 5

The existence of internal aqueous volume in the particles of the complex formed between the block copolymers and surfactants of the present invention can be conveniently demonstrated using a water-soluble  
35 fluorescent dye, 5,6-carboxyfluorescein. See, for example, Parker, Photoluminescence of Solutions;

- 40 -

Elsevier: New York, p.303 et seq., 1968. The complex between N-cetylpyridinium bromide and polyethylene oxide-block-poly(sodium methacrylate) is prepared as described in Example 3 at  $C_t/C_i = 1$  replacing the  
5 solution of the copolymer in water for the solution of the same copolymer in 10 mM of 5,6-carboxyfluorescein (Sigma Co.). Briefly, 0.87 ml of the aqueous solution of 10 mM 5,6-carboxyfluorescein, pH 7.4 is mixed with 50  $\mu$ l of 0.039 M solution of polyethylene  
10 oxide-block-poly(sodium methacrylate) and 80  $\mu$ l of 0.025 M N-cetylpyridinium bromide solution. After 30 minutes incubation at room temperature the particles of the complex were separated from the dye remaining in the external volume by gel permeation  
15 chromatography using Sephadex G-25 medium (Pharmacia Biotech) equilibrated with the solution of 0.01 mM N-cetylpyridinium bromide. The fluorescence emission of 5,6-carboxyfluorescein in the solutions obtained is determined at the excitation wavelength 495 nm and  
20 250°C using Shimadzu 5000 spectrofluorimeter. The maximum of fluorescence emission of the dye in this system was observed at 517.2 nm (slit 1.5 nm) which corresponds to the emission maximum of the free dye in the absence of the particles. Addition of 40  $\mu$ l of  
25 10% (v/v) aqueous Triton X-100 (Sigma Co.) to this system results in a sharp increase in the fluorescence intensity resulting from the release of the concentrated dye from the internal aqueous cavity of the particles. This suggests that the particles  
30 represent the vesicles with an internal aqueous volume.

EXAMPLE 6

35 The complex between N-cetylpyridinium bromide and polyethylene oxide-block-poly(sodium methacrylate) was prepared as described in Example 5

replacing 10 mM 5,6-carboxyfluorescein solution with 0.1 mM solution of calcein (Sigma Co.). The particles of this complex were separated by gel permeation chromatography as described in Example 5. The 5 fluorescence emission intensity of calcein was determined at the emission and excitation wavelengths 490 nm and 520 nm respectively. 2  $\mu$ l of  $\text{CoCl}_2$  solution and 40  $\mu$ l of 10% Triton X-100 were added to this system in different order to quench fluorescence 10 outside the vesicles. Addition of  $\text{CoCl}_2$  resulted in the decrease of the fluorescence in this system to 3 % of the initial fluorescence. When Triton X-100 was added after  $\text{CoCl}_2$ , the fluorescence was further decreased to 1 % of the initial value. In contrast, when Triton X-100 15 was added without prior addition of  $\text{CoCl}_2$ , no change in the fluorescence was recorded. When  $\text{CoCl}_2$  solution was added after the Triton X-100 solution the fluorescence was decreased to about 1 % of the initial value. This suggests formation of the vesicles with an internal 20 volume of about 2 % of the total volume.

#### EXAMPLE 7

The complex between N-cetylpyridinium bromide and polyethylene oxide-block-poly(sodium 25 methacrylate) is prepared as described in Example 5 replacing 10 mM 5,6-carboxyfluorescein solution with 1  $\mu$ M solution of alkaline phosphatase (Sigma Co.). The vesicles are separated from the external solution by gel permeation chromatography using Sephadex G-25 30 medium (Pharmacia Biotech) equilibrated with the solution of 0.01 mM N-cetylpyridinium bromide. The amount of alkaline phosphatase incorporated into the vesicles is determined enzymatically using p-nitrophenyl phosphate (Sigma Co.) as a substrate 35 (Larsson, Immunochemistry: Theory and Practice, CRC Press, Boca Raton, FL, 1988). This assay reveals that

- 42 -

about 1 % of protein incorporates into the vesicles.

EXAMPLE 8

5       The complex between N-cetylpyridinium bromide and polyethylene oxide-block-poly(sodium methacrylate) is prepared as described in Example 2 replacing 10 mM 5,6-carboxyfluorescein solution for 1  $\mu$ M solution of acridin-labeled 20-mer phosphodiester oligonucleotide synthesized as described by  
10      Vinogradov, et al., Biochem. Biophys. Res. Commun. 1994, 203: 959. The fraction of oligonucleotide incorporated into the vesicles determined by acridine is 75 % of initial.

15

EXAMPLE 9

18      A polyethyleneglycol (8,000)-polyethylene imine (m.w. 2,000) copolymer was synthesized as previously reported by Vinogradov et al. (Pharm. Res., 14: S-641).

20

18      A complex between the polyethylene glycol-polyethyleneimine copolymer and anionic surfactant, Aerosol OT (Sigma Co.) was obtained at  $C_t/C_i = 1$ , where  $C_t$  is the total concentration of added surfactant, and  $C_i$  is the concentration of ionic groups 25 of the block copolymer. The size of the particles formed, determined by dynamic light scattering as described in Example 3, above equals 57 nm.

EXAMPLE 10

30

18      The complex between the polyethylene glycol-polyethyleneimine copolymer, exemplified above, and fatty acid salt, oleic acid sodium salt (Sigma Co.) was obtained at  $C_t-C_i=1$  in sodium phosphate buffer (SPB), 10 mM, pH 6.0 by simple mixing of the copolymer solution in the same buffer and fatty acid salt solution in methanol. The final content of methanol

- 43 -

in the complex solution was 3%. The size of the particles formed, determined by dynamic light scattering as described in Example 3, above, was 54 nm.

5

EXAMPLE 11

The complex between the polyethylene glycol-polyethylenimine copolymer, mentioned above, and fatty acid salt, oleic acid sodium salt was obtained at  $C_t - C_i = 1$  in sodium phosphate buffer (SPB), 10 mM, pH 7.4, as described in Example 10, above. The size of the particles formed, determined by dynamic light scattering as described in Example 3, above, was 44 nm.

15

EXAMPLE 12

The complex between the polyethylene glycol-polyethylenimine copolymer, mentioned above, and fatty acid salt, oleic acid sodium salt was obtained at  $C_t - C_i = 1$  in TRIS-buffer, 10 nM, pH 8.2, as described in Example 10, above. The size of the particles formed, determined by dynamic light scattering as described in Example 3, above, was 56 nm.

25

EXAMPLE 13

The complex between the polyethylene glycol-polyethylenimine copolymer and retinoic acid (Aldrich Co.) was obtained at  $C_t - C_i = 1$  in sodium phosphate buffer (SPB), 10 mM, pH 7.4. The solution of retinoic acid in water/methanol (60:40, v/v) mixture containing sodium hydroxide was added to the copolymer solution in the SPB. The final content of methanol in the complex solution was 5%. The additional absorption band ( $\lambda_{max} = 306$  nm) in the UV-vis spectrum of retinoic acid in the presence of copolymer was found to be comparable to that of pure retinoic acid at the same

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conditions ( $\lambda_{\max}=343$  nm).

**EXAMPLE 14**

42.8 mg of nonafluoropentanoic acid (Aldrich  
5 Co.) and 1 mg of Taxol were mixed in 50  $\mu$ l of ethanol  
containing sodium hydroxide ( $2.84 \times 10^{-4}$  mole). This  
mixture was added to 1 ml of the complex between the  
polyethylene glycol-polyethyleneimine copolymer and  
oleic acid sodium salt prepared as described in  
10 Example 10, above. After stirring overnight, the  
sample was centrifuged 10 minutes at 13000 RPM. 20  $\mu$ l  
of supernatant was added to 1 ml of methanol and UV  
spectra were recorded. The Taxol concentration was  
calculated from the absorbency at 227 nm ( $\epsilon=44,359$   
15 ml/mg). The extinction coefficient was estimated in  
the presence of fluoroorganic component in methanol.  
The degree of Taxol solubilization was 74.4%. The  
size of the complex particles loaded with the  
Taxol/Fluoroorganic compound mixture determined by  
20 dynamic light scattering as described in Example 3,  
above, was 61 nm.

While certain embodiments of the  
present invention have been described and/or  
25 exemplified above, various other embodiments will be  
apparent to those skilled in the art from the  
foregoing disclosure. The present invention is,  
therefore, not limited to the particular embodiments  
described and/or exemplified, but is capable of  
30 considerable variation and modification without  
departure from the scope of the appended claims.

## WHAT IS CLAIMED IS:

1. A composition comprising a therapeutic or diagnostic agent and a supramolecular complex, said complex comprising as constituents (i) a block copolymer, having at least one nonionic, water soluble segment and at least one polyionic segment, and (ii) at least one charged surfactant having hydrophobic groups, the charge of said surfactant being opposite to the charge of the polyionic segment of said block copolymer, the constituents of said complex being bound by interaction between said opposite charges and between surfactant hydrophobic groups, and with the proviso that when said therapeutic or diagnostic agent is an ionic substance having a net charge opposite to the charge of said block copolymer, the net charge of said therapeutic or diagnostic agent is no more than 10.

2. A composition as claimed in claim 1, wherein said therapeutic or diagnostic agent comprises about 0.1 to about 99.9 weight percent of said composition.

3. A composition as claimed in claim 1, wherein the ratio of the net charge of said surfactant to the net charge of the polyionic segment present in said block copolymer constituent of said complex is between about 0.01 and about 100.

4. A composition as claimed in claim 3, wherein said charge ratio is between about 0.1 and about 10.

5. A composition as claimed in claim 3, wherein the polyionic segment of said block copolymer is polyanionic.

5                   6. A composition as claimed in claim 5,  
wherein the nonionic segment of said block copolymer  
is selected from the group consisting of  
polyetherglycols, copolymers of ethylene oxide and  
propylene oxide, polysaccharides, and homopolymers and  
10 copolymers of vinyl compounds selected from the group  
consisting of acrylamide, acrylic acid esters,  
methacrylamide, methacrylic esters, N-(2-  
hydroxypropyl)methacrylamide, vinyl alcohol, vinyl  
pyrrolidone, vinyl triazole, or the N-oxide of  
15 vinylpyridine, polyorthoesters and polyamino acids.

7. A composition as claimed in claim 5,  
wherein said polyanionic segment is selected from the  
group consisting of polymethacrylic acids and its  
salts, polyacrylic acid and its salts, copolymers of  
methacrylic acid and its salts, copolymers of acrylic  
acid and its salts, heparin, poly(phosphate),  
polyamino acid, polymaleic acid, polylactic acid,  
nucleic acid or carboxylated dextran.

25                   8. A composition as claimed in claim 5,  
wherein said polyanionic segment is a homopolymer or a  
co-polymer prepared from a monomer which polymerizes  
to form a product with carboxyl pendant groups, said  
monomer being selected from the group consisting of  
30 acrylic acid, asparatic acid (amino acid), 1,4-  
phenylenediacrylic acid citraconic acid, citraconic  
anhydride, trans cinnamic acid, 4-hydroxy-3-methoxy  
cinnamic acid, p-hydroxy cinnamic acid, trans-  
35 glutaconic acid, glutamic acid (amino acid), itaconic  
acid, linoleic acid, linolenic acid, methacrylic acid,

maleic acid, maleic anhydride, mesaconic acid, trans- $\beta$ -hydromuconic acid, trans-trans muconic acid, oleic acid, ricinoleic acid, 2-propene-1-sulfonic acid, 4-styrene sulfonic acid, trans-traumatic acid,  
5 vinylsulfonic acid, vinyl phosphate acid, vinyl benzoic acid, vinyl glycolic acid.

9. A composition as claimed in claim 5,  
wherein said surfactant is selected from the group  
10 consisting of lipophilic quaternary ammonium salts,  
lipopolyamines, lipophilic polyamino acids, lipophilic primary-, secondary-, tertiary- and heterocyclic amines, lipophilic imidazoles, lipophilic piperidinium salts, lipophilic quinaldinium salts, lipophilic  
15 azonium and azonium salts, pH-sensitive cationic lipids, dicationic bolaform electrolytes or a mixture of said surfactants.

10. A composition as claimed in claim 5,  
20 further including a nonionic surfactant.

11. A composition as claimed in claim 10,  
wherein said nonionic surfactant is selected from the group consisting of dioleoyl phosphatidylethanolamine,  
25 dioleoyl phosphatidylcholine, or a mixture of said nonionic surfactants.

12. A composition as claimed in claim 3,  
wherein the polyionic segment of said block copolymer  
30 is polycationic.

13. A composition as claimed in claim 12,  
wherein said polycationic segment is selected from the group consisting of polyamino acid, alkanolamine  
35 esters of polymethacrylic acid, polyamines, polyalkyleneimines, polyvinyl pyridine and quaternary

ammonium salts of said polycationic segments.

14. A composition as claimed in claim 12, comprising an anionic surfactant selected from the  
5 group consisting of alkyl sulfates, alkyl sulfonates, fatty acid soaps, salts of hydroxy-, hydroperoxy-, polyhydroxy-, epoxy- fatty acids, salts of mono- and polycarboxylic acids, prostanoic acid and prostaglandines, leukotriens and lipoxines, alkyl  
10 phosphates, alkyl phosphonates, lipids, sodium-dialkyl sulfosuccinate, n-alkyl ethoxylated sulfates, cholate and desoxycholate of bile salts, perfluorocarboxylic acids, fluoroaliphatic phosphonates, fluoroaliphatic sulphates.

15

15. A composition as claimed in claim 1, in the form of vesicles, said vesicles having an internal volume containing said therapeutic or diagnostic agent.

20

16. A composition as claimed in claim 1, wherein said therapeutic or diagnostic agent is a charged species, having a positive or negative charge.

25

17. A composition as claimed in claim 17, wherein said therapeutic or diagnostic agent is a constituent of said supramolecular complex.

30

18. A composition as claimed in claim 1, wherein said therapeutic or diagnostic agent is selected from the group consisting of analgesic agents, anti-inflammatory agents, antibacterial agents, antiviral agents, antifungal agents, antiparasitic agents, tumorcidal or anti-cancer agents, proteins, toxins, enzymes, hormones, neurotransmitters, glycoproteins, immunoglobulins, immunomodulators,

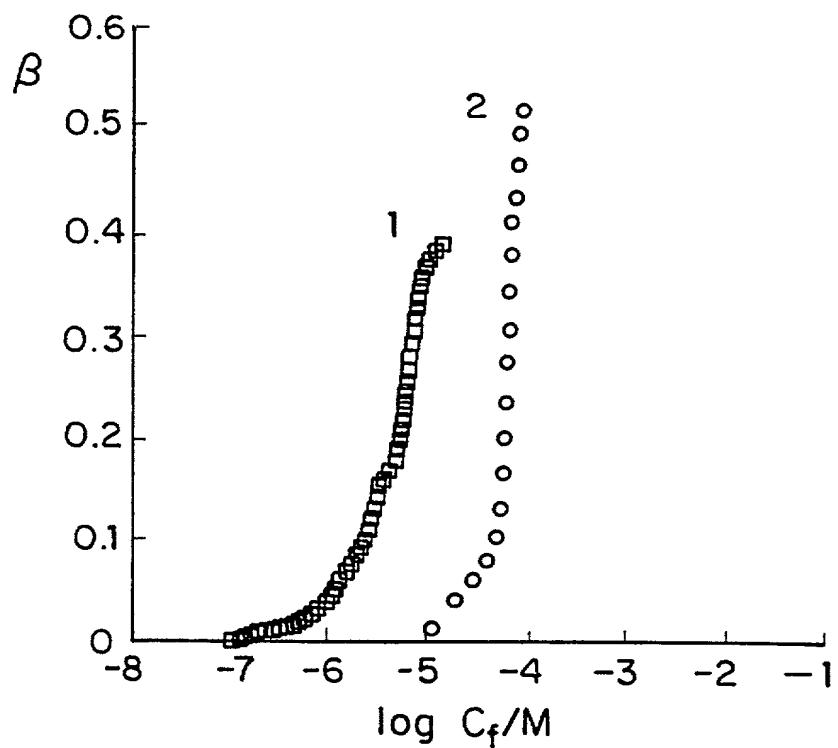
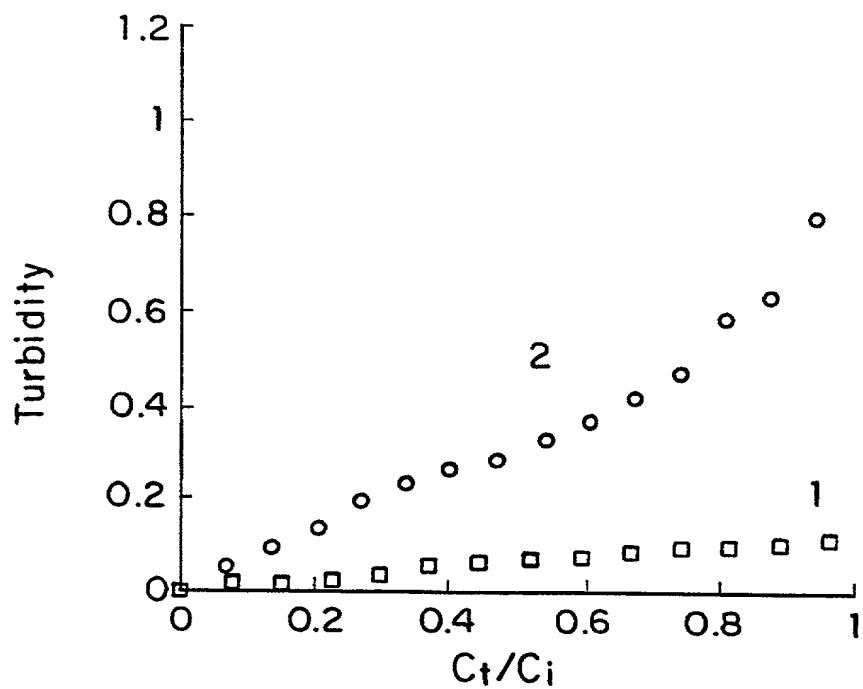
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dyes, radiolabels, radio-opaque compounds, fluorescent compounds, polysaccharides, cell receptor binding molecules, anti-glaucomic agents, mydriatic compounds and local anesthetics.

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**FIG. 1****FIG. 2**

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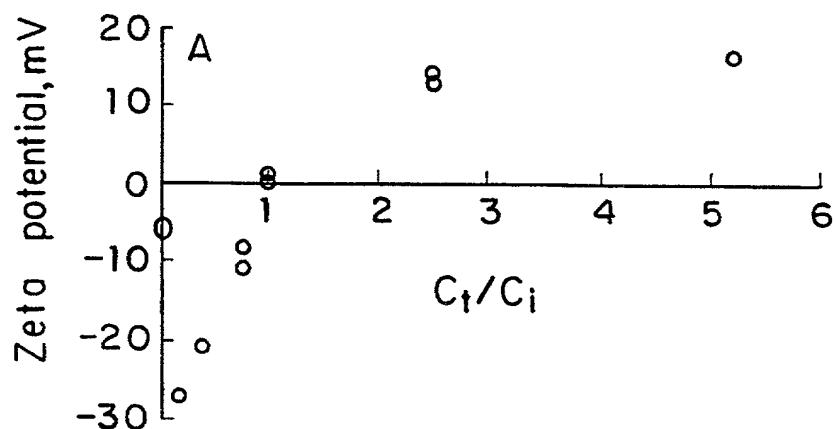


FIG. 3A

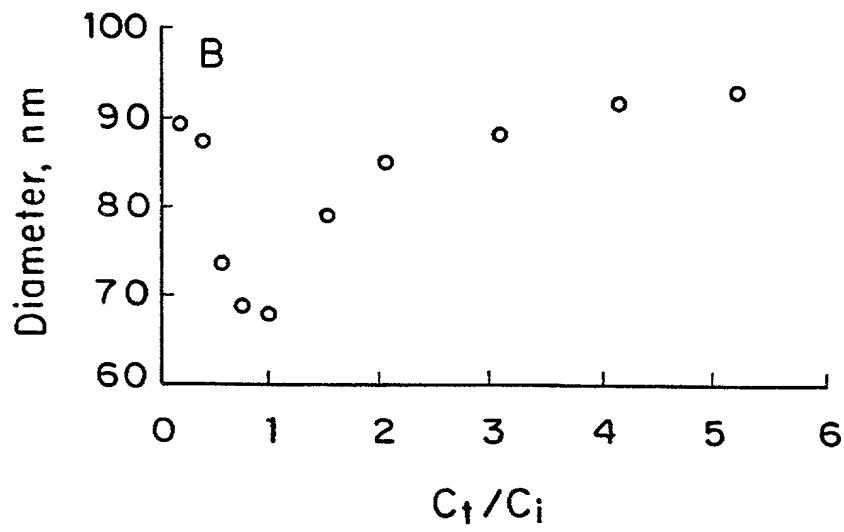
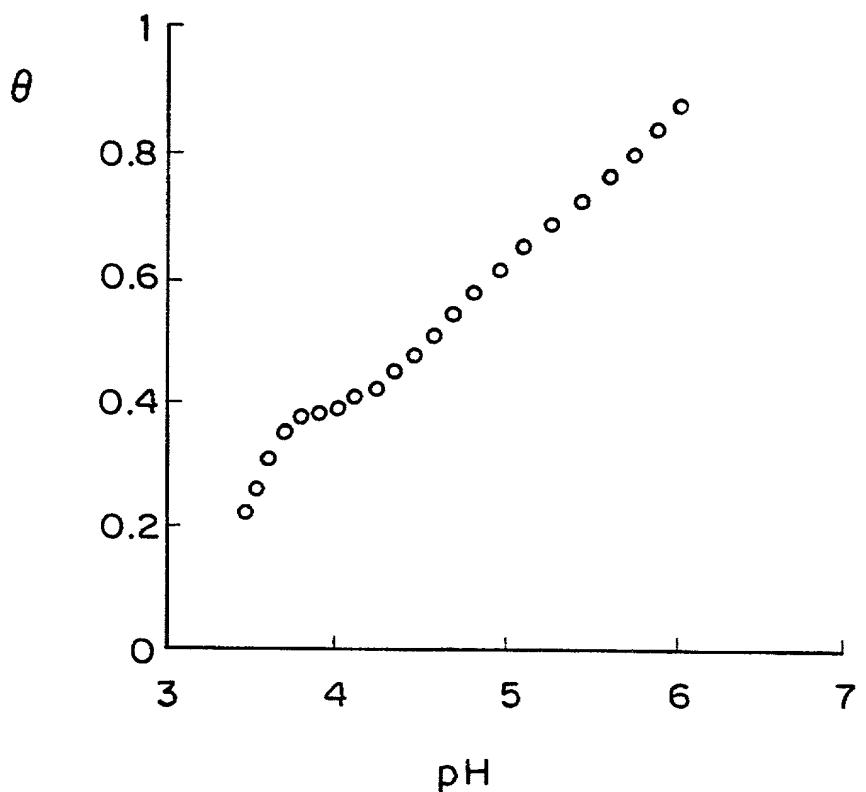


FIG. 3B

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**FIG. 4**



## DECLARATION, POWER OF ATTORNEY AND POWER TO INSPECT

As a below named inventor, I hereby declare:

that my residence, post office address and citizenship are as stated below next to my name;

that I verily believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the invention entitled: **COMPOSITIONS FOR DELIVERY OF BIOLOGICAL AGENTS AND METHODS FOR THE PREPARATION THEREOF**

the specification of which [check one(s) applicable]

was filed 11 June 1998 as International Application No. PCT/US98/12138 [now U.S. Application No. 09/445,653]  
 and was amended by Amendment filed \_\_\_\_\_ (if applicable); [or];  
 is attached to this Declaration, Power of Attorney and Power to Inspect;

that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; and

that I acknowledge my duty to disclose information which is material to the examination of this application in accordance with Rule 56(a) [37CFR§1.56(a)].

**CLAIM UNDER 35 USC §119(e):** I hereby claim the benefit under 35 USC §119(e) of any United States provisional applications listed below:

<u>Provisional Application No.</u>	<u>Filing Date</u>
	<u>Day/Mo/Year</u>
60/049,552	13 June 1997

**POWER OF ATTORNEY:** As inventor, I hereby appoint **DANN, DORFMAN, HERRELL AND SKILLMAN, P.C.** of Philadelphia, PA, and the following individual(s) as my attorneys or agents with full power of substitution to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: **Patrick J. Hagan, Reg. No. 27,643** and **Kathleen D. Rigaut, Ph.D., Reg. No. 43,047.**

**POWER TO INSPECT:** I hereby give **DANN, DORFMAN, HERRELL AND SKILLMAN, P.C.** of Philadelphia, PA or its duly accredited representatives power to inspect and obtain copies of the papers on file relating to this application.

**SEND CORRESPONDENCE TO: CUSTOMER NUMBER 000110.**

**DIRECT INQUIRIES TO:** Patrick J. Hagan, Esq.  
Telephone: (215) 563-4100 Facsimile: (215) 563-4044

Thereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

### 1-10 SOLE OR FIRST JOINT INVENTOR

Full Name Alexander V. KABANOV  
First Middle Last

Signature Alexander Kabanov

Date 08. 16. 00

Residence Omaha, Nebraska NE  
City State or Country

Citizenship Russia

Post Office Address:  
1304 South 126<sup>th</sup> Street

Omaha, Nebraska 68144  
City State or Country Zip Code

### 3-10 THIRD JOINT INVENTOR (IF ANY)

Full Name Victor A. KABANOV  
First Middle Last

Signature \_\_\_\_\_

Date \_\_\_\_\_

Residence Moscow, Russia Russia  
City State or Country

Citizenship Russia  
Post Office Address:  
Lomonosovsky Prospect 14, Apt. 18

Moscow, Russian Federation 117296

### 2-10 SECOND JOINT INVENTOR (IF ANY)

Full Name Adi EISENBERG  
First Middle Last

Signature \_\_\_\_\_

Date \_\_\_\_\_

Residence Montreal, Canada CA  
City State or Country

Citizenship U.S.A.

Post Office Address:  
6100 Deacon Road, Apt. 15J

Montreal, Canada PQ H3S2V6  
City State or Country Zip Code

### FOURTH JOINT INVENTOR (IF ANY)

Full Name \_\_\_\_\_  
First Middle Last

Signature \_\_\_\_\_

Date \_\_\_\_\_

Residence \_\_\_\_\_  
City State or Country

Citizenship \_\_\_\_\_  
Post Office Address:  
\_\_\_\_\_

# DECLARATION, POWER OF ATTORNEY AND POWER TO INSPECT

As a below named inventor, I hereby declare:

that my residence, post office address and citizenship are as stated below next to my name;

that I verily believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the invention entitled: **COMPOSITIONS FOR DELIVERY OF BIOLOGICAL AGENTS AND METHODS FOR THE PREPARATION THEREOF**

the specification of which [check one(s) applicable]

was filed 11 June 1998 as International Application No. PCT/US98/12138 [now U.S. Application No. 09/445,653]  
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DIRECT INQUIRIES TO: **Patrick J. Hagan, Esq.**  
Telephone: (215) 563-4100 Facsimile: (215) 563-4044

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

## SOLE OR FIRST JOINT INVENTOR

Full Name Alexander V. KABANOV  
First Middle Last

Signature \_\_\_\_\_

Date \_\_\_\_\_

Residence Omaha, Nebraska  
City State or Country

Citizenship Russia  
Post Office Address:  
1304 South 126<sup>th</sup> Street

Omaha, Nebraska 68144  
City State or Country Zip Code

## THIRD JOINT INVENTOR (IF ANY)

Full Name Victor A. KABANOV  
First Middle Last

Signature \_\_\_\_\_

Date \_\_\_\_\_

Residence Moscow, Russia  
City State or Country

Citizenship Russia  
Post Office Address:  
Lomonosovsky Prospect 14, Apt. 18

Moscow, Russian Federation 117296

## SECOND JOINT INVENTOR (IF ANY)

Full Name Adi EISENBERG  
First Middle Last

Signature Adi Eisenberg

Date March 23 80

Residence Montreal, Canada  
City State or Country

Citizenship U.S.A.  
Post Office Address:  
6100 Deacon Road, Apt. 15J

Montreal, Canada PQ H3S2V6  
City State or Country Zip Code

## FOURTH JOINT INVENTOR (IF ANY)

Full Name \_\_\_\_\_  
First Middle Last

Signature \_\_\_\_\_

Date \_\_\_\_\_

Residence \_\_\_\_\_  
City State or Country

Citizenship \_\_\_\_\_  
Post Office Address:  
\_\_\_\_\_

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As & below named Inventor, I, V. Kabanov, do declare:

that my residence, post office address and citizenship are as stated below next to my name;

that I verily believe I am the original, first and sole Inventor (if only one name is listed below) or an original, first and joint Inventor (if two or more names are named below) of the invention entitled: **COMPOSITIONS FOR DELIVERY OF BIOLOGICAL AGENTS AND METHODS FOR THE PREPARATION THEREOF**

the specification of which (check one(s) applicable)

 was filed 11 June 1998 as International Application No. PCT/US98/12136 (now U.S. Application No. 09/645,655)and was amended by Amendment filed 11 June 1998 (if applicable); (or): is attached to this Declaration, Power of Attorney and Power to Inspect;

that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; and

that I acknowledge my duty to disclose information which is material to the examination of this application in accordance with Rule 58(a) (37 CFR 1.56(a)).

CLAIM UNDER 35 USC §119(e): I hereby claim the benefit under 35 USC §119(e) of any United States provisional applications listed below

Provisional Application No.Issue DateDay/Month/Year

60/048,962

13 June 1997

**POWER OF ATTORNEY:** As Inventor, I hereby appoint DANN, DORFMAN, HERRELL AND SKILLMAN, P.C. of Philadelphia, PA, and the following individual(s) as my attorney or agents with full power of substitution to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Patrick J. Hagan, Reg. No. 27,843 and Kathleen G. Mignot, Ph.D., Reg. No. 43,047.**POWER TO INSPECT:** I hereby give DANN, DORFMAN, HERRELL AND SKILLMAN, P.C. of Philadelphia, PA or its duly accredited representative power to inspect and obtain copies of the papers on file relating to this application.**SEND CORRESPONDENCE TO:** CUSTOMER NUMBER 000110.**DIRECT INQUIRIES TO:** Patrick J. Hagan, Esq.

Telephone: (215) 383-4100 Facsimile: (215) 383-4044

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of my application or any patent issued thereon.

**SOLE OR FIRST JOINT INVENTOR**Full Name Alexander V. KABANOV  
First Middle Last

Signature \_\_\_\_\_

Date \_\_\_\_\_

Residence Omaha, Nebraska  
City State or CountryCitizenship Russia  
Post Office Address: 1304 South 12th StreetOmaha, Nebraska 68149  
City State or Country Zip Code**THIRD JOINT INVENTOR (IF ANY)**Full Name Victor A. KABANOV  
First Middle LastSignature V. KabanovDate July 03, 2000Residence Moscow, Russia  
City State or CountryCitizenship Russia  
Post Office Address: Lomonosovskiy Prospekt 14, Apt. 18Moscow, Russian Federation 117296**SECOND JOINT INVENTOR (IF ANY)**Full Name Adi EISENBERG  
First Middle Last

Signature \_\_\_\_\_

Date \_\_\_\_\_

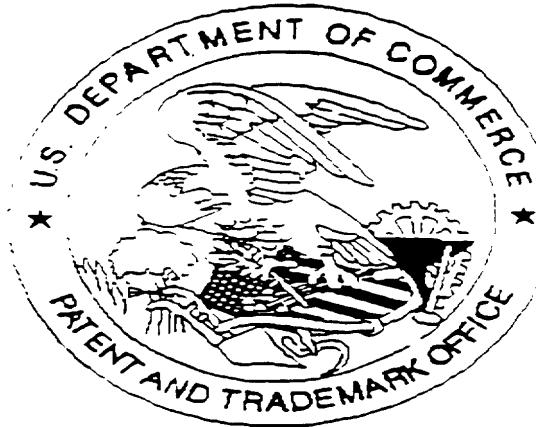
Residence Montreal, Canada  
City State or CountryCitizenship U.S.A.  
Post Office Address: 6100 Dawson Road, Apt. 151Montreal, Canada PO H3S 2V6  
City State or Country Zip Code**FOURTH JOINT INVENTOR (IF ANY)**Full Name   
First Middle Last

Signature \_\_\_\_\_

Date \_\_\_\_\_

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